ACTA PATHOLOGICA
T MICROBIOLOGICA
SCANDINAVICA

REDACTORES

REDIGENDA CURAVIT

J CHR SIIM
København

VOL 77 1969

MUNKSGAARD COPENHAGEN 1969

Munksgaard
International Booksellers and Publishers Ltd
47 Prags Boulevard DK 2300 Copenhagen S Denmark



PRINTED IN DENMARK
VALD. PEDERSENS BOGTRYKKERI
COPENHAGEN

INDEX

VOL 77 FASC 1–4 1969

Acta path microbiol scand 77 1–23 1969

The Immunological Laboratory Statens Seruminstitut
Copenhagen Denmark

THE FUNCTION OF THE IMMUNE RESPONSE IN TUMOUR GROWTH AND ITS GENETICAL REGULATION

A new Hypothesis

By

J V SPARCK

Received 4 ii 69

Tumour transplantation is one of the best experimental systems available for the study of the biological mechanisms underlying cancer It must be realized however that it is an artificial situation that may be complicated by factors not entering a spontaneous tumour host system Extrapolations from transplantation tumours to primary tumours should therefore only be made provided that the transplantation system used represents the closest approximation possible to the natural tumour phenomenon In the light of present knowledge of the genetics and the immunology of tissue transplantation this would mean that only a primary spontaneous neoplasm transferred to a recipient genetically identical with the donor can be considered a useful experimental model Few investigations have fulfilled this demand

It seems to be an essential characteristic of the tumour phenomenon that the organism in which it develops is not immunized against its own tumour cells Very many transplantation experiments show that a spontaneous tumour transplanted to a syngeneic recipient cannot release a rejection reaction According to the definition generally accepted by transplantation immunologists the tissue of the spontaneous tumour is therefore non antigenic to the host in the case of genetic identity between donor and host In contrast the transplantation of tumour between a donor and a recipient which are genetically non identical generally results in a rejection response and in the immunization of the host against the transplanted tumour The immunity induced in that case is however an expression of general transplantation immunity due to tissue antigens also present in the normal tissues of the donor

In spite of these well established immunological relationships the assumption is nevertheless widespread that tumours in general are

Requests for reprints should be addressed to Dr J V Sparck Immunological Laboratory Statens Seruminstitut Amager Boulevard 80 2300 Copenhagen S Tel ASTA 2817 lokal 468

foreign to their hosts with respect to specific antigens Reports are available concerning the demonstration in certain experimental tumour systems (including chemically and virally induced neoplasms) of tumour specific antigens capable of inducing responses in genetically compatible hosts also (for review see *Old & Boyse* 1964, *Hellstrom & Moller* 1965) Apparently this kind of tumour growth takes place in spite of an immune reaction against it i e a reaction which does not result in destruction of the tumour The idea has therefore spread that generally the development of a tumour is connected with a defect in the immune response of the host organism

The papers in which evidence of tumour specific antigens has been demonstrated have been concerned with experimentally induced neoplasms and the question could be raised to what extend they are representative of the spontaneous tumour It is a definite possibility which cannot be excluded that the antigenicity found is a feature particular to certain forms of artificial carcinogenesis

The experiments reported in this paper show that an antagonistic relationship between the growth of the tumour and the immune responsiveness of the host does not exist under all circumstances In the murine tumour system investigated it was found that provided the transplantation concerned a primary spontaneous tumour and no immunogenetic disparity the tumour growth was positively related to the reactive capacity of the host and was not dependent on an immunosuppression

MATERIALS AND METHODS

Mouse Strains

The C3H/Ssc line of the Statens Seruminstitut had been maintained by strict single line brother sister mating as were the two other strains used This C3H line shows a high incidence of spontaneous mammary carcinomas which occur in females from about 8–9 months of age With respect to H 2 antigens the C3H mice are of the H 2k type

The AKR/Ssc line which is a Danish subline of the AKR strain has never shown any spontaneous mamma carcinomas This line is also of the H 2k type

The DBA/2 Ssc used were of the H 2d type

The mice were 2½–3½ months of age and between 20 and 25 g in weight when used as recipients of tumour grafts

Tumour Transplantation

Primary mammary tumours were used which had arisen spontaneously in C3H or DBA females of 10–20 months of age Histologically these were adenocarcinomas exhibiting a basic acinar structure This kind of spontaneous mammary tumour was never seen to regress spontaneously

In order to graft identical quantities of tumour material to different recipients transplantations were always made with single cell suspensions The carcinoma was removed from its site under the skin The tissue was trimmed free of necrotic parts cut into small fragments with scissors and washed in Gey s balanced salt solution (*Gey & Gey* 1936) containing 50 IU of penicillin and 50 mcg of streptomycin per ml The tissue fragments were then forced through a fine mesh d wire screen and the suspension finally filtered The cell number was counted in a haemocytometer and the percentage of living cells was judged by supravital staining with 0 1 per cent eosin The suspensions used for transplantation usually contained between 30 and 40 per cent cells unstained by eosin indicating that propor

tion to be living The usual inoculum was 0 1 ml adjusted to contain a total of 10^6–10^7 cells

The spontaneous C3H and DBA carcinomas used for transplantation normally caused progressive growth in all syngeneic recipients of both sexes and never regressed spontaneously They never took in allogeneic recipients The tumour growth obtained was always only local and metastases were never found The development of the tumour was followed twice a week by measuring the two main diameters of each tumour with calipers The average diameter of the group at different times was then computed In earlier experiments (*Sparel* 1962) tumour weights were also determined and it was found that these were proportional to the diameters to the third power

Irradiation

Irradiation was carried out at the Radiophysical Laboratory Radium Centre Copenhagen as described in previous reports (*Sparel* 1961)

Cortisone Treatment

Cortisone treatment was carried out with the Shering Corporation Bloomfield New Jersey USA cortisone acetate preparation This is an aqueous suspension containing 50 mg/ml of 11 dehydro 17 hydroxy cortico sterone acetate Injections were made subcutaneously on the back of the mouse to avoid the region of the flank where tumour inoculations were made

Antiserum Production

Specific antisera for the cyt toxic test were produced by immunizing C3H mice and DBA mice against DBA and C3H tissues respectively Antigen for one injection of 20 mice (6 month old males) was prepared by collecting the spleen liver lungs and kidneys from a 1 year old mouse of the foreign strain This material was minced in Gey s solution washed once and resuspended in 4 ml solution For the first experiment sera were produced by giving four 0 2 ml injections intraperitoneally to each of 20 mice at intervals of 48 hours and 3 weeks later a fifth injection In the later experiments tissue antigen was prepared in the same way but was suspended in 2 ml of Gey s solution which was emulgated in 2 ml of Freund s complete adjuvant Each of 20 mice was immunized by a subcutaneous injection of 0 2 ml of this emulgation made from the foreign strain and 5 weeks later a similar intraperitoneal injection without Freund s adjuvant In all the immunizations the mi e were bled from the tail 8 days after the last injection and again the next day from the heart The sera from each group of 20 mice were collected and pooled and stored at 4 C

Cytotoxic Antibody Test

The serological technique for the demonstration of antigens in tumour tissue was based on the method *Gorer* & *O Gorman* (1956) Equal volumes of a cell suspension in Gey s solution of about 2×10^7 cells/ml and of antiserum dilution were incubated at 37 C for 15–30 minutes The same volume of complement (guinea pig serum) was added to the reaction mixture and incubation continued for a further 40 minutes One drop of 0 1 per cent eosin or 0 1 per cent trypan blue solution was then added The number of damaged (stained) and undamaged (unstained) cells was determined by direct microscopy of drops of suspension placed on slides in rings of vaseline with cover slips Cell suspensions incubated with saline served as controls The cytotoxic index (*Hellstrom* 1959) was calculated as

$$\frac{\text{percentage unstained in control—percentage unstained in test}}{\text{percentage unstained in control}}$$

RESULTS

Effect of Whole Body Irradiation of Recipients on the Growth of Syngeneic and Allogeneic Tumours

Fig 1 illustrates the tumour growth obtained following the subcutaneous implantation of a primary spontaneous C3H mammary

foreign to their hosts with respect to specific antigens Reports are available concerning the demonstration in certain experimental tumour systems (including chemically and virally induced neoplasms) of tumour specific antigens capable of inducing responses in genetically compatible hosts also (for review see *Old & Boyse* 1964 *Hellstrom & Moller* 1965) Apparently this kind of tumour growth takes place in spite of an immune reaction against it *i e* a reaction which does not result in destruction of the tumour The idea has therefore spread that generally the development of a tumour is connected with a defect in the immune response of the host organism

The papers in which evidence of tumour specific antigens has been demonstrated have been concerned with experimentally induced neoplasms, and the question could be raised to what extend they are representative of the spontaneous tumour It is a definite possibility which cannot be excluded that the antigenicity found is a feature particular to certain forms of artificial carcinogenesis

The experiments reported in this paper show that an antagonistic relationship between the growth of the tumour and the immune responsiveness of the host does not exist under all circumstances In the murine tumour system investigated it was found that provided the transplantation concerned a primary spontaneous tumour and no immunogenetic disparity the tumour growth was positively related to the reactive capacity of the host and was not dependent on an immunosuppression

MATERIALS AND METHODS

Mouse Strains

The C3H/Ssc line of the Statens Seruminstitut had been maintained by strict, single line brother sister mating as were the two other strains used This C3H line shows a high incidence of spontaneous mammary carcinomas which occur in females from about 8–9 months of age With respect to H 2 antigens the C3H mice are of the H 2k type.

The AKR/Ssc line which is a Danish subline of the AKR strain has never shown any spontaneous mamma carcinomas This line is also of the H 2k type

The DBA/2 Ssc used were of the H 2d type

The mice were 2½–3½ months of age and between 20 and 25 g in weight when used as recipients of tumour grafts

Tumour Transplantation

Primary mammary tumours were used which had arisen spontaneously in C3H or DBA females of 10–20 months of age Histologically these were adenocarcinomas exhibiting a basic acinar structure This kind of spontaneous mammary tumour was never seen to regress spontaneously

In order to graft identical quantities of tumour material to different recipients transplantations were always made with single-cell suspensions The carcinoma was removed from its site under the skin. The tissue was trimmed free of necrotic parts cut into small fragments with scissors and washed in Gey s balanced salt solution (*Gey & Gey* 1936) containing 50 IU of penicillin and 50 mcg of streptomycin *per ml* The tissue fragments were then forced through a fine meshed wire screen and the suspension finally filtered The cell number was counted in a haemocytometer and the percentage of living cells was judged by supravital staining with 0 1 per cent eosin The suspensions used for transplantation usually contained between 30 and 40 per cent cells unstained by eosin indicating that propor

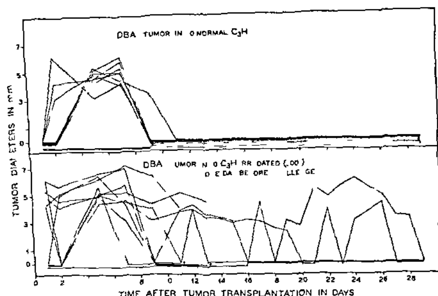


Fig 3

Effect of whole body X irradiation on allogeneic tumour transplantation The growth curve of each individual tumour is shown

carcinoma in normal *syngeneic* mice and in syngeneic mice irradiated 52 days previously with either 550 or 750 R The latter dose is lethal and the mice exposed to that dose were therefore protected by intra venous injections of about 2 × 10 syngeneic foetal liver cells

It will be seen that the treatment resulted in reduced tumour growth in the irradiated recipients and that this effect of irradiation was dose dependent

Further experiments concerning the time factor demonstrated that the radiation effect obtained in this system of syngeneic tumour graft ing was not dependent on radiation having taken place before trans plantation Fig 2 shows that in a group given 550 R irradiation 27 days *after* tumour challenge an initially high growth rate is reduced imme diately after the X ray treatment and subsequently is the same as in a group irradiated 20 days *before* grafting In other words the inhib itory effect of irradiation on the development of tumours does not only affect the initial establishment of the syngeneic tumour graft but also the growth of already established tumours

Fig 2 also illustrates a characteristic of the tumour growth observed consistently following the transplantation of syngeneic carcinomas It can be seen that the tumour development is a two phase phenom enon *viz* moderate initial growth reaching its maximum about one week after grafting followed by a temporary regression and a sub sequent progressive growth phase in this case starting after the 15th day The time interval between the two phases may vary and in some

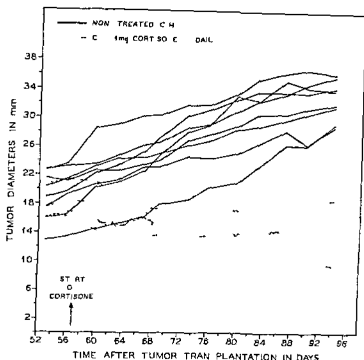
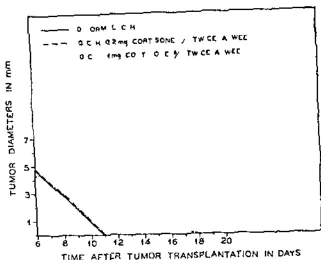


Fig 6

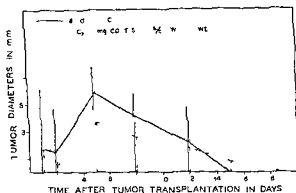
Growth curves for individual tumours which developed after the implantation of a primary spontaneous C3H mamma carcinoma (tumour 642) in syngeneic recipients It can be seen how the treatment with cortisone stops the further progression of these tumours

grafting and thereafter of two weekly injections of 1 mg for 4 months Fig 5 shows the tumour growth in this experiment It can be seen that in the normal group the progressive tumour growth begins about the 19th day after challenge and by about 2 months later all the recipients had succumbed The survival range of this group was 42–91 days after tumour grafting and the mean survival time 65 5 days In the treated group tumours grew out in all animals at the same time as in the untreated group but at a much slower rate By about 40 days after grafting the tumours had reached a level which was maintained with only slight fluctuations for the next 2–3 months The survival range was 59–173 days after grafting and the mean survival time 110 days In this experiment also the treatment caused emaciation and weakness

Furthermore it was demonstrated that cortisone inhibits the growth not only of newly established tumours but also of tumours established long before treatment began Fifteen normal C3H females all had mammary tumours following the implantation 57 days previously of a primary spontaneous C3H carcinoma Seven of these mice were then given a daily subcutaneous injection of 1 mg of cortisone while the remaining eight animals were left untreated Fig 6 illustrates the



a



b

Fig 7

Effect of cortisone on the tumours developed after the implantation of cells from two allogeneic mamma carcinomas (a DBA tumour 660 b DBA tumour 694) in C3H mice The curves represent average tumour diameter in the groups ranges (in b) are indicated by vertical lines The effect of treatment is a moderately prolonged persistence of tumours as well as a depression of initial out growth

growth curves of all the 15 tumours from the 53rd day after tumour challenge and onwards for 37 days It can be seen that all the tumours in the non treated animals progressed uninterruptedly while all those in the animals given cortisone stopped progressing immediately after injections were initiated

Experiments on the effect of cortisone on grafts of primary *allogeneic* mamma carcinomas were also carried out Cells from a spontaneous DBA mammary tumour were used but otherwise the procedure was exactly the same as in the experiment on syngeneic transplantation described in Fig 4 overleaf Fig 7a shows that grafting of the allogeneic

tumour cells gave rise to the formation of small tumours in all three groups. In the normal recipients these tumours reached their maximum (twice the size of the corresponding initial tumours after syngeneic grafting) at about the 5–6th day after inoculation and then regressed rapidly, rejection being completed after 8–11 days. The animals given the low dosage treatment showed no obvious reduction in their rejection response. However, in the high dosage group the rejection was clearly delayed. Growth continued after the 6th day, five of ten tumours persisted on the 14th day and rejection in this group was not completed until after 14–18 days.

It is apparent that the immuno suppression by cortisone did not result in any actual enhancement of the tumour growth. Although a prolonged persistence was obtained, there was no increase in tumour size but rather a depression. This can be seen more clearly from Fig. 7b which shows the result of a similar experiment. In this case also the treatment with cortisone caused a delay in the rejection response as shown by a moderately prolonged persistence of the tumours. However, in addition a slight repression of the initial tumour growth was found in the recipients treated with cortisone.

The results of the cortisone experiments described are in good agreement with the irradiation results reported above. Both kinds of experiments lend support to the view that growth which results from a syngeneic tumour graft requires a certain host reaction. The different effect of immuno suppression on the tumour growth obtained with different immuno genetic relationship between donor and recipient indicates that it is the mode of the host response, regulated by antigenic factors of the transplant, which is decisive for tumorous development.

Analysis of the Antigenic Composition of Tumours Developed Following Transplantation to Chimaeric or Hybrid Recipients

The observation of a positive correlation between the host response and the tumour growth consistently found following compatible grafting justifies an examination of the extend to which the host tissue contributes to the growing neoplasm. To investigate this problem two experimental systems were used.

In one system, radiation chimaeras were employed as recipients, i.e. lethally irradiated (750 R) mice of one inbred strain protected after irradiation by the injection of 2 × 10 foetal haematopoietic (liver) cells of another inbred strain. Mice *syngeneic* to the irradiated recipients were used as tumour donors. It was assumed that a system was thus obtained in which the tumour graft was unrejectable but in which the graft and host elements could still be destinguished on certain antigenic criteria. If the developing tumour partly or wholly owed its origin to the primitive host reticular tissue, the implanted

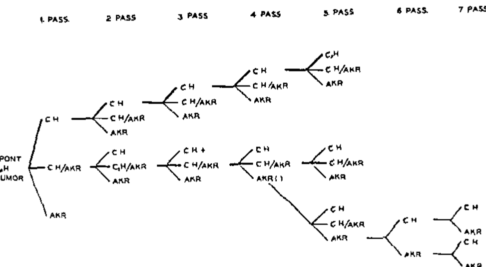


Fig 8

Diagram illustrating the transplantation scheme used for the analysis of the different transplantability characteristics of two different transplantation lines of a carcinoma which occurred in a C3H mouse One line was maintained by serial passage *via* normal C3H mice the other by serial passage *via* C3H/AKR chimaeras

allogeneic component in the host mesenchyme would serve as host marker

The other experimental system made use of F_1 hybrids between two inbred strains of mice as recipients of tumour grafts from one of the parental strains Again the homozygous tumour is unrejectable but in this situation the host specific marker is a normal constituent in a normal organism and not an artificially implanted component in an irradiated animal

In the first experiments investigation of the transplantability characteristics of tumours developing in chimaeric organisms was made according to the plan shown in Fig 8 The approach was to transplant subcutaneously a primary spontaneous C3H mamma carcinoma as cell suspension to three different groups of six male recipient mice normal C3H allogeneic chimaeras C3H/AKR (irradiated C3H mice protected by implant of AKR foetal liver cells) and normal AKR In the latter group there was no take but immediate regression while there was growth in C3H and C3H/AKR After the first passage the tumour could now be passed serially by two different sublines a C3H line and a chimaera line The former was established by transferring a tumour of the first transplant passage in a C3H mouse to the same three kinds of recipients and so on making each new passage from normal C3H hosts The other line was started by grafting a first passage tumour from a C3H/AKR animal to the three types of recipients and making each new passage from a tumour growing in a

tumour cells gave rise to the formation of small tumours in all three groups In the normal recipients these tumours reached their maximum (twice the size of the corresponding initial tumours after syngeneic grafting) at about the 5–6th day after inoculation and then regressed rapidly rejection being completed after 8–11 days The animals given the low dosage treatment showed no obvious reduction in their rejection response However in the high dosage group the rejection was clearly delayed Growth continued after the 6th day five of ten tumours persisted on the 14th day and rejection in this group was not completed until after 14–18 days

It is apparent that the immuno suppression by cortisone *did not result in any actual enhancement* of the tumour growth Although a *prolonged persistence was obtained there was no increase in tumour size* but rather a depression This can be seen more clearly from Fig 7b which shows the result of a similar experiment In this case also the treatment with cortisone caused a delay in the rejection response as shown by a moderately prolonged persistence of the tumours However in addition a slight repression of the initial tumour growth was found in the recipients treated with cortisone

The results of the cortisone experiments described *are in good agreement* with the irradiation results reported above Both kinds of experiments lend support to the view that growth which results from a syngeneic tumour graft requires a certain host reaction The different effect of immuno suppression on the tumour growth obtained with different immuno genetic relationship between donor and recipient indicates that it is the mode of the host response regulated by *antigenic factors of the transplant* which *is decisive for tumorous* development

Analysis of the Antigenic Composition of Tumours Developed Following Transplantation to Chimaeric or Hybrid Recipients

The observation of a *positive correlation between the host response* and the tumour growth consistently found following compatible grafting justifies an examination of the extend to which the host tissue contributes to the growing neoplasm To investigate this problem two experimental systems were used

In one system radiation chimaeras were employed as recipients *i e* lethally irradiated (750 R) mice of one inbred strain protected after irradiation by the injection of 2×10^7 foetal haematopoietic (liver) cells of another inbred strain Mice *syngeneic* to the irradiated recipients were used as tumour donors It was assumed that a system was thus obtained in which the tumour graft was unrejectable but in which the graft and host elements could still be destinguished on certain antigenic criteria If the developing tumour partly or wholly owed its origin to the *primitive host reticular tissue* the implanted

brids and the other to 10 homozygous C3H and 12 DBA×C3H hybrids All the 40 resulting tumours were analysed and it was found that they reacted quite uniformly according to their group Table 3 shows the results of about half of these tumours with anti DBA serum (from C3H mice) and anti C3H serum (from DBA mice) The reactions with normal spleens and spontaneous tumours are shown uppermost together with first passage C3H tumours in C3H recipients It can be seen that the sera *employed are highly strain specific with respect* to cytotoxic activity Furthermore the cell suspension from C3H × DBA hybrids are equally reactive with both kinds of antisera This applies not only to the spleen suspensions but also to the suspensions of tumours developed after the grafting of C3H carcinomas This indicates that at least about 50 per cent of such transplantation tumours are built up of cells derived from the host

TABLE 3

Cytotoxic Action of Anti DBA and Anti C3H Sera on Tumour Cells of C_3HxDBA F_1 Hybrids given Grafts of C_3H Tumours

Cell suspension	Days after implant of C_3H carcin	% unstained (surviv) cells after incub with saline or 1 5 dil antiserum: saline (control)	anti DBA (serum 2)	anti C_3H (serum 3)
Norm DBA spleen	–	95	7 (0 93)	95 (0)
Norm C_3H spleen	–	9?	92 (0)	7 (0 92)
Spont DBA carcin		52	3 (0 94)	53 (< 0)
Spont C_3H carcin 35	–	48	50 (< 0)	7 (0 86)
C_3H carcin 35 1st pass C_3H	18	51	48 (0 06)	11 (0 78)
DBAxC_3H F_1 No 1? (carcin 35)	18	52	10 (0 81)	12 (0 77)
(spleen)	18	95	2 (0 98)	4 (0 96)
No 15 (carcin 35)	31	54	6 (0 88)	7 (0 87)
No 13 (carcin 35)	34	50	9 (0 8?)	10 (0 80)
No 18 (carcin 35)	38	48	2 (0 95)	7 (0 85)
(spleen)	38	93	4 (0 96)	6 (0 94)
" No 14 (carcin 35)	45	92	3 (0 97)	6 (0 93)
No 17 (carcin 35)	53	51	3 (0 94)	8 (0 84)
No 2? (carcin 35)	55	51	3 (0 94)	15 (0 71)
No 19 (carcin 35)	61	51	9 (0 82)	4 (0 92)
Spont C_3H carcin 32	–	54	49 (0 09)	8 (0 85)
C_3HxDBA F_1 No 10 (carcin 32)	48	52	5 (0 90)	2 (0 96)
No 4 (carcin 32)	51	61	6 (0 90)	7 (0 89)
No ? (carcin 32)	52	48	8 (0 83)	6 (0 88)
(spleen)	52	92	5 (0 95)	1? (0 89)
No 8 (carcin 32)	5?	51	? (0 8?)	11 (0 78)
(spleen)	5?	9	4 (0 95)	11 (0 88)
" No 1? (carcin 32)	57	54	7 (0 86)	8 (0 85)
No 18 (carcin 32)	71	48	7 (0 85)	10 (0 79)
No 13 (carcin 32)	72	46	5 (0 89)	7 (0 85)
No 1 (carcin 32)	77	42	2 (0 95)	9 (0 79)
(spleen)	77	94	4 (0 96)	16 (0 83)

Each percentage is based on the counting of ?00 cells
Figures in brackets represent cytotoxic indices

The observations concerning the change of transplantability and antigenic composition of tumours produced by transplants from homozygous C3H donors to chimaeric or hybrid recipients emphasize the importance of the host reaction for development of transplantation tumours The incorporation of immunogenetic characteristics of the host into the tumour suggests a more active participation of the connective tissue of the host in the formation of the tumour than is usually accepted

DISCUSSION AND CONCLUSION

The aim of the present investigation was to gain more insight into the role of immunological reactions of the host organism in the growth of transplanted tumours

In order to provide a sufficiently well defined experimental system to justify comparison with the natural tumour phenomenon only spontaneous mamma carcinomas from strictly inbred strains of mice were used for transplantation The results of whole body X irradiation or cortisone administration to the recipients of tumour grafts show quite clearly that the effect of a depression of the immune response is a reduced tumour growth provided that donor and recipient are of identical genotype On the other hand if the donor host combination involves genetical differences the effect of immuno suppression is the reverse *i e* the tumour which normally should regress within a certain time persists longer before the final regression takes place

The present observations seem to indicate that when the genetical conditions make the development of tumour possible the tumour growth is not a function of a defect in the immune response of the host organism On the contrary the greater the defect in the immune response the lesser the tumour growth Obviously these spontaneous tumours cannot immunize their primary hosts nor syngeneic recipients The fact that the reaction of the host does not have an antagonistic effect on the growth of tumour is evidence that the tumour in the present system is not equipped with any tumour specific antigens

The basic problem of the origin of the tissue of the tumour from either the cells of the graft or from the host has hitherto been left unexamined The reported immuno genetical analysis of transplantation tumours using chimaeric or hybrid recipients of homozygous grafts showed that the cells of the tumour had host properties These results thus indicate that the tumour is to a great extend derived from the host

Most previous experimental work on the relation of the immune response to the growth of transplanted tumours has been at variance with the present findings and hypothesis A number of early reports

of specific tumour immunity must be considered unacceptable because the experiments were conducted with animal material of unproven genetical homogeneity or with tumours which had been transplanted for very long periods (for review see *Hauschka* 1952). More recent studies based on a better understanding of the immunogenetics of tissue transplantation have been carried out with genetically homogeneous animal strains. A number of reports on the experimental demonstration of tumour immunity are available. The more substantial cases seem to be restricted to some chemically induced and some virally induced tumours. The first more clearcut demonstration was that of *Foley* (1953) who reported that the removal of a growing transplant of a tumour recently induced by methylcolanthrene (MC) in an inbred strain of mice was followed by some resistance to subsequent challenge with the same tumour, while a similar removal of a transplant of a spontaneous tumour did not result in any immunity. The demonstration of an induced immunity to MC induced tumours in syngeneic or even primary hosts was confirmed by *Prehn & Main* (1957), *Reves* (1960), *Klein et al* (1960) and *Old et al* (1962). Similar results were obtained with benzpyrene induced tumours (*Feldman et al* 1963).

A striking fact concerning the chemically induced tumours is the lack of a common antigenicity as shown by the rarity of immunological cross reactivity between the individual tumours. This is not true of the other class of experimental neoplasms against which a state of tumour immunity has been convincingly demonstrated, viz. virus induced tumours. The existence of specific antigens in a number of tumours of known viral aetiology, including those induced with polyoma virus have been reported (*Sjogren et al* 1961, *Habel* 1962), Gross virus (*Klein et al* 1962) and Moloney virus (*Sachs* 1962, *Klein & Klein* 1965). It is characteristic that tumours induced by the same oncogenic virus have the same specific antigens and therefore cross react in immunization.

In contrast to the above mentioned results with chemically or virally induced tumours, attempts to demonstrate a specific antigenicity of spontaneous tumours have generally failed.

It is true that Weiss and co-workers (*Weiss et al* 1966, *De-Julian et al* 1967) have reported the demonstration of tumour associated immunogenicity expressed in murine mammary tumours as well as in preneoplastic and normal mammary tissues of mice infected with mammary tumour virus (MTV). Experiments on the effect of various immunological activators and of *immunization* of recipients with tumour tissue or normal tissue gave variable results, sometimes heightened resistance and *sometimes* tumour enhancement being obtained. However, the carcinomas used by *Weiss et al* can hardly be considered spontaneous in the strict sense since most of them had been serially transplanted beyond the first transplant generation.

The observations concerning the change of transplantability and antigenic composition of tumours produced by transplants from homozygous C3H donors to chimaeric or hybrid recipients emphasize the importance of the host reaction for development of transplantation tumours The incorporation of immunogenetic characteristics of the host into the tumour suggests a more active participation of the connective tissue of the host in the formation of the tumour than is usually accepted

DISCUSSION AND CONCLUSION

The aim of the present investigation was to gain more insight into the role of immunological reactions of the host organism in the growth of *transplanted* tumours

In order to provide a sufficiently well defined experimental system to justify comparison with the natural tumour phenomenon only *spontaneous mamma carcinomas from strictly inbred strains of mice* were used for transplantation The results of whole body X irradiation or cortisone administration to the recipients of tumour grafts *show quite clearly that the effect of a depression of the immune* response is a reduced tumour growth provided that donor and recipient are of identical genotype On the other hand if the donor host combination involves genetical differences the effect of immuno suppression is the reverse *i e* the tumour which normally should regress within a certain time persists longer before the final regression takes place

The present observations seem to indicate that when the genetical conditions make the development of tumour possible the tumour growth is not a function of a defect in the immune response of the host organism On the contrary the greater the defect in the immune response the lesser the tumour growth Obviously these spontaneous tumours cannot immunize their primary hosts nor syngeneic recipients The fact that the reaction of the host does not have an antagonistic effect on the growth of tumour is evidence that the tumour in the present system is not equipped with any tumour specific antigens

The basic problem of the origin of the tissue of the tumour from either the cells of the graft or from the host has hitherto been left unexamined The reported immuno genetical analysis *of transplantation* tumours using chimaeric or hybrid recipients of homozygous grafts showed that the cells of the tumour had host properties These results thus *indicate that the tumour is to a great extend derived from* the host

Most previous experimental work on the relation of the immune response to the growth of transplanted tumours has been at variance with the present findings and hypothesis A number of early reports

of specific tumour immunity must be considered unacceptable because the experiments were conducted with animal material of unproven genetical homogeneity or with tumours which had been transplanted for very long periods (for review see *Hauschka* 1952) More recent studies based on a better understanding of the immuno genetics of tissue transplantation have been carried out with genetically homo geneous animal strains A number of reports on the experimental demonstration of tumour immunity are available The more substantial cases seem to be restricted to some chemically induced and some virally induced tumours The first more clearcut demonstration was that of *Foley* (1953) who reported that the removal of a growing transplant of a tumour recently induced by methylcolanthrene (MC) in an inbred strain of mice was followed by some resistance to sub sequent challenge with the same tumour while a similar removal of a transplant of a spontaneous tumour did not result in any immunity The demonstration of an induced immunity to MC induced tumours in syngeneic or even primary hosts was confirmed by *Prehn & Main* (1957) *Revesz* (1960) *Klein et al* (1960) and *Old et al* (1962) Similar results were obtained with benzpyrene induced tumours (*Feldman et al* 1963)

A striking fact concerning the chemically induced tumours is the lack of a common antigenicity as shown by the rarity of immunological cross reactivity between the individual tumours This is not true of the other class of experimental neoplasms against which a state of tumour immunity has been convincingly demonstrated *viz* virus induced tumours The existence of specific antigens in a number of tumours of known viral aetiology including those induced with polyoma virus have been reported (*Sjogren et al* 1961 *Habel* 1962) Gross virus (*Klein et al* 1962) and Moloney virus (*Sachs* 1962 *Klein & Klein* 1965) It is characteristic that tumours induced by the same oncogenic virus have the same specific antigens and therefore cross react in immunization

In contrast to the above mentioned results with chemically or virally induced tumours attempts to demonstrate a specific antigenicity of spontaneous tumours have generally failed

It is true that *Weiss* and co workers (*Weiss et al* 1966 *DeOme et al* 1967) have reported the demonstration of tumour associated immunogenicity expressed in murine mammary tumours as well as in preneoplastic and normal mammary tissues of mice infected with mammary tumour virus (MTV) Experiments on the effect of various immunological activators and of immunization of recipients with tumour tissue or normal tissue gave variable results sometimes heightened resistance and sometimes tumour enhancement being obtained However the carcinomas used by *Weiss et al* can hardly be considered spontaneous in the strict sense since most of them had been serially transplanted beyond the first transplant generation

Evidence is available (*Morton* 1964 *Nowinski et al* 1968) that the transmission of virus from the mother or neonatal infection in some virus induced mouse tumour systems renders the mice immunologically tolerant to tumour virus or tumour antigen Therefore one might suggest the possibility that the retarded tumour growth observed in syngeneic recipients given X rays or cortisone is due to a termination of tolerance caused by this treatment However in the present experiments the reduced tumour growth in immuno suppressed hosts cannot be an effect of breaking a state of immunological tolerance to a tumour agent The tumour repressing effect of the treatment described is an immediate one It is inconceivable that a treatment which causes a general suppression of immune responsiveness could simultaneously result in an immediate restoration of immune reactivity to an agent to which the organism was tolerant beforehand

It should be emphasized that the antigenic patterns found in tumours induced by certain artificial agents are not necessarily in force in the natural tumour phenomenon Apart from the observations in the present study several reports are available which indicate that the tumour formation is dependent in a positive way on an efficient host response *Hollcroft et al* (1951) found that X irradiation of a transplanted mouse lymphosarcoma (of the same mouse strain) gave only a slight regression effect if the irradiation (1300 R) was only local whereas the regression effect was much increased if the same X ray dose was distributed so that the tumour got 1000 R locally and the rest of the body 300 R *Vasiliev* (1958) showed that pre irradiation of rats inhibited the growth of tumour transplants of the same strain while heterospecific (mouse) grafts were enhanced A similar irradiation effect on the syngeneic tumour transplantation in mice was reported by *Sparck* (1961) who also found a retarded growth of tumour grafts in radiation chimaeras irrespective of the source of the cells used for reconstitution of the irradiated animal Observations by *Gross & Schober* (1960) also support the view that the development of tumours is dependent on the state of the lymphoid tissue A large number of rats given whole body X irradiation were examined histologically and it was found that the occurrence of neoplasms was inversely correlated with the degree of radiation damage to the lymphoid organs

The view that inflammation and connective tissue proliferation favour tumour growth is also supported by several previous demonstrations of a suppressive effect of cortisone on the progressive growth of transplanted tumours in certain donor host combinations (*Heilman & Kendall* 1944 *Schober* 1952 *Antopol Glaubach & Graff* 1954 *Baserga & Shubik* 1954 *Selye* 1955 *Vasiliev* 1958) There are also a great number of observations to the effect that intensified local inflammation facilitates the establishment and promotes growth of tumour grafts (*Jones & Rous* 1914 *Zahl & Nowak* 1949 *Selye* 1957 *Vasiliev* 1958)

There is pronounced agreement between the findings reported in the present communication and the results of studies by *Wheatley* and co workers (*Wheatley & Ambrose* 1964 *Wheatley & Easty* 1964) who used the invasion of transplanted mouse ascites tumour into the peritoneum increase in body weight and ascitic fluid accumulation as evidence of tumour development They found that infiltration of the peritoneum of the host always precedes invasion and that suppression of host immune responsiveness by treatment with cortisone whole body irradiation or thymectomy reduce tumour development On the other hand the more intense the response of the host *e g* through stimulation with irritants the more rapid the tumour development The results reported in the present paper suggest that details of the immunogenetic relationship between donor and host decide in each particular case whether immuno suppression causes retardation or promotion of the tumour growth Although the transplantation of Ehrlich s ascites tumour can hardly be considered equal to the syngeneic grafting of a spontaneous tumour it might by chance meet the same immunogenetic requirements

The other part of the present study which concerns the antigenic composition of tumours growing in chimaeric or hybrid hosts touches on the important problem of the origin of the cells which make up the transplanted tumour In other words does a tumour grow only by proliferation of its own cells or do host cells turn into tumour cells? In transplantations in which the tumour graft was genetically compatible to but distinguishable from the host it was clearly demonstrated that the majority of cells from the growing tumour had the immunogenetic properties of host cells It would of course be expected that the host contributes a supporting stroma to the tumour and evidence has been published indicating a decisive role of the stroma in tumour transplantability Particularly the extensive studies by *Greene* (1957) on heterospecific transplantations have stressed the importance of the antigenic components of the host stroma for the future transplantability of the tumour

In the present study it was possible to recover up to about 50 per cent viable cells in a tumour cell preparation and to demonstrate by direct serological testing that practically all reacted as host cells It is very difficult to interpret this as merely an effect of stroma contamination It is unlikely that the high percentage of living cells recovered represents only stroma cells (morphological distinction was not possible since all cells in the dissociated form appeared as round cells)

It seems to be a more acceptable interpretation of the findings that the tumours in the transplantation system studied are formed by an inflammatory proliferation of the host mesenchyme which is transformed into tumorous structures Histological examinations of the same transplantation system lend support to this view (*Sparck & Gross*

1969) The findings in the present investigation are not explained in a satisfactory way by the commonly accepted ideas of tumorogenesis but justify a hypothesis interpreting the tumour growth as a consequence of the basic self non self discrimination which is a necessity for the maintenance of a multicellular organism Material which is foreign i e antigenic *must* cause a rejection response whereas material not sufficiently foreign *cannot* release a rejection response The results show that the suppression of the lymphoreticular system of the recipient had opposite effects on the tumour growth in the case of allogeneic and syngeneic tumour donors This is in agreement with the concept of the neoplastic process as a particular reaction modus of the host mesenchyme determined by a certain immunogenetical relationship of the components of tumour and host If the tumour possesses no constituents antigenic to the host the latter is deprived of the ability to complete a rejection and therefore the inflammatory proliferation provoked cannot be terminated and results in a tumorous development In that case the effect of suppressed reactivity should be a depressed tumour growth

The suggested immunological mechanism may well be the basis of many forms of neoplastic growth It is reasonable to anticipate the occurrence of variant tissue constituents which are neither in sufficient agreement with the normal constituents to be eliminated by the normal degradation mechanism nor sufficiently antigenic to be rejected Such agents will function as irritants which activate an inflammatory response of the local tissue without subsequent restoration of the tissue equilibrium

This conception of the neoplastic process as a reaction phenomenon would not be in conflict with the well established laws of transplantation genetics The histocompatibility genes which have been found to segregate in a Mendelian way determine the formation of tissue antigens but obviously these genes determine simultaneously the reaction modus to certain challenges The rules of transplantability will be exactly *the same whether the transplanted tumour cells actually* multiply in the compatible genotype or whether, as suggested here a constituent of the tumour is transferred which for immunogenetic reasons cannot be rejected and thus stimulates the host mesenchyme to progressive proliferation resulting in a tumour

SUMMARY

Studies on the role of the immunological reactions of the host organism in the growth of transplanted tumours are reported An experimental system was used which consisted of strictly inbred C3H mice as recipients of mammary carcinomas from syngeneic (C3H) or allogeneic (DBA) donors In order to secure well defined genetical relations only primary spontaneous tumours were transplanted

Tumours were implanted subcutaneously as cell suspensions and the development of tumours was recorded. Whole body X irradiation and cortisone injections were used as methods for reducing the immunological responsiveness of the recipients.

These experiments have shown that the effect of a depression of the immune response is a reduced tumour growth provided that the donor and recipient are of identical genotype. If genetical differences are involved in the donor host combination the effect of immuno suppression is the reverse viz. a prolonged persistance of tumour before the final regression. These findings indicate that when the genetical conditions make the development of tumour possible the tumour growth is not a function of a defect immune response of the host.

In continuation of this demonstration experiments were carried out to elucidate the problem whether the cells of the transplantation tumour originate from the cells of the graft or from the host. By using recipient mice which were either chimaeras of two strains of mice or hybrids between them and homozygous tumour donors from one of the strains an experimental system was obtained in which the tumour would grow but in which cells of donor and host origin could be distinguished on antigenic markers. Both by transplantability tests and by a serological technique it could be shown that the tumour in this system acquired the properties of the host. It was found by the direct cytotoxic test that practically all the recovered cells reacted as host cells.

The immunological and genetical findings reported suggest a hypothesis which explains the tumour formation as a reaction phenomenon viz. a particular form of inflammatory proliferation of the host mesenchyme which is transformed into tumorous structures.

REFERENCES

Antopol W, Glaubach S & Graff S Retardation of growth of implants of carcinoma 755 in cortisone injected mice. Proc Soc exp Biol (N Y) *86* 364–366 1954

Baserga R & Shubik P The action of cortisone on transplanted and induced tumours in mice. Cancer Res *14* 12–16 1954

De Ome K B, Lavrin D H, Shen A, Blair P B & Weiss D W Immunology of spontaneous mammary carcinomas in mice. Studies on the nature of the protective antigens. In Carcinogenesis (Symp Fund Cancer Res 1966) Williams & Wilkins Co Baltimore, Md 1967

Feldman M, Globerson A & Yaffe D Immunological properties of chemically induced sarcomas. Acta Union Int Cancer *19* 65–72 1963

Foley E J Antigenic properties of methylcolanthrene induced tumors in mice of the strain of origin. Cancer Res *13* 835–837 1953

Gey G O & Gey M C Maintenance of human normal cells and tumor cells in tissue culture. Amer J Cancer *27* 45–6 1936

Gorer P A & O'Gorman P The cytotoxic activity of isoantibodies in mice. Transplant Bull *3* 142–143 1956

Greene H S N Heterotransplantation of tumors Ann N Y Acad Sci *69* 818–829 1957
Gross K & Schober B Occurrence of tumors in rats surviving X irradiation (in Czech) Neoplasma Suppl 1960 pp 23–30
Habel K Immunological determinants of polyoma virus oncogenesis J exp Med *115* 181–193 1962
Hauschka T S Immunologic aspects of cancer A review Cancer Res *12* 615–633 1952
Heilman F R & Kendall E C The influence of 11 dehydro 17 hydroxy cortico sterone (compound E) on the growth of a malignant tumour in the mouse Endocrinol *34* 416–420 1944
Hellstrom K E Cytotoxic effect of isoantibodies on mouse tumor cells *in vitro* Transplant Bull *6* 411 416 1959
Hellstrom K E & Moller G Immunological and immunogenetic aspects of tumor transplantation Progr Allergy *9* 158–245 1965
Hollcroft J Lorenz E & Hunstiger H Effects of ionizing radiations on a trans planted lymphosarcoma J nat Cancer Inst *11* 1–16 1950
Jones F S & Rous P On the cause of the localization of secondary tumours at points of injury J exp Med *20* 404–412 1914
Klein E & Klein G Antigenic properties of lymphomas induced by the Moloney agent J nat Cancer Inst *32* 547–568 1964
Klein G Sjögren H O Klein E & Hellstrom K E Demonstration of resistance against methylcolanthrene induced sarcomas in the primary autochtonous host Cancer Res *20* 1561–1572 1960
Klein G Sjogren H O & Klein E Demonstration of host re istance against iso transplantation of lymphomas induced by the Gross agent Cancer Res *22* 955–961 1962
Morton D L Acquired immunological tolerance to spontaneous mammary adeno carcinomas following neonatal infection with mammary tumor agent (MTA) Proc Amer Ass Cancer Res 5 46 1964
Nowinski R C Boyse F A Old L J & Carswell E A Influence of mammary tumor virus infection on the acceptance or rejection of transplanted ML + leukemias Proc Soc exp Biol (N Y) *127* 20–25 1968
Old L J Boyse F A Clarke D A & Carswell E A Antigenic properties of chemi cally induced tumors Ann N Y Acad Sci *101* 80 106 1962
Old L J & Boyse E A Immunology of experimental tumors Ann Rev Med *15* 167–186 1964
Prehn R T & Main J M Immunity to methylcolanthrene induced sarcomas J nat Cancer Inst *18* 769–778 1957
Révész L Detection of antigenic differences in isologous host tumor systems by pretreatment with heavily irradiated tumor cells Cancer Res *20* 443–451 1960
Sachs L The transplantability of an X ray and a virus induced leukemia in iso logous mice inoculated with a leukemia virus J nat Cancer Inst *29* 759–764 1962
Selye H The effect of antiphlogistic corticoid conditioning (the A CC effect) upon the Walker tumor Cancer Res *15* 26 30 1955
Selye H Effect of inflammation upon the growth of transplantable neoplasms a demonstrated by the double granuloma pouch technique Brit J Cancer *11* 550–553 1957
Sjögren H O Hellstrom I & Klein G Transplantation of polyoma virus induced tumors in mice Cancer Res *21* 329–337 1961
Sparck J V Inhibition of transplants of spontaneous isologous tumors in mouse radiation chimaeras Acta path microbiol scand *51* 403–415 1961
Spärck J V Immunity and host response in the growth of transplanted tumour Munksgaard Copenhagen 1962
Spärck J V & Gross K The role of the host mesenchyme in the development of tumours after transplantation Acta path microbiol scand *77* 24–38 1969
Vasiliev J M Connective tissue reaction in homologous and heterologic tumour transplantation (In Russian with an English summary) Voprosy Onkologii *4* 11–17 1958
Weiss D W Lavrin D H De Julian M Yaage J & Blair P B Studies on the immunology of spontaneous mammary carcinomas of mice *In Viruses in*

ducing cancer (W J Burdette Ed) Univ of Utah Press Salt Lake City Utah 1966

Wheatly D N & Ambrose F J Tumour cell invasion from transplantable ascites tumours into host tissue Brit J Cancer *18* 730-742 1964

Wheatly D N & Easty C C The growth and infiltration of Ehrlich's ascites tumour in mice with reduced immunological responses Brit J Cancer *18* 743-755 1964

Zahl P A & Nowak A Effect of subcutaneous injury on tumour growth in the mouse Proc Soc exp Biol (N Y) *70* 266-272 1949

Acta path microbiol scand 77 24-38 1969

The Immunological Laboratory Statens Seruminstitut Copenhagen and Fibiger Laboratory Kgs Lyngby Denmark

THE ROLE OF THE HOST MESENCHYME IN THE DEVELOPMENT OF TUMOURS AFTER TRANSPLANTATION

By

J V SPARCK and KAREL GROSS[1]

Received 4 ii 69

It is a general assumption that the growth of a spontaneous tumour is based upon the local proliferation of a cell variant which has acquired autonomy and is able to multiply independent of the normal tissue regulations Correspondingly a tumour which develops following the transplantation of neoplastic tissue from a donor of adequate genotype is thought to be built up mainly by the autonomous growth of the implanted tumour cell variant However it is often forgotten that the evidence for this assumption is only indirect and circumstantial

A number of observations suggest that other interpretations might be considered It is now widely accepted that the connective tissue of the host has got important functions in relation to the development of both spontaneous and transplanted tumours It is accepted that an inflammatory response of the host provides the tumour with a vascular supply and supporting stroma and a metabolic environment favouring tumour proliferation has been shown to depend on a stimulated host connective tissue (*Vasiliev* 1958 *Whealley & Ambrose* 1964) However the problem as to whether the connective tissue reaction of the host has functions beyond those mentioned deserves more attention In spite of its obvious importance it has been left largely unexamined *to what extent cells of the host connective tissue enter into and directly* contribute to the formation of the tumour tissue

Long standing clinicopathological observations on processes of hyperplasia and tumour formation in man (*Gross* 1953 1964) have shown that these processes always begin as an activation and proliferation of the mesenchymal cells and are closely related to the momentary state of the local connective tissue

Recent experimental work on immune status and tumour growth is in accordance with the observations just mentioned It was shown (*Sparck* 1961 1962 1969) that when spontaneous mammary tumours

---

[1] Present address Institute of Pathology University of Lund Sweden

in mice were grafted to syngeneic recipients the depression of the immune and connective tissue reactions by treatment with X rays or cortisone consistently resulted in a strong reduction of the tumour growth This implies that in case of a genetic identity between the donor and the recipient the tumour growth is dependent in a positive way on the state of the host mesenchyme Furthermore it could be shown in certain parent to hybrid transplantations that the cells of the transplantation tumour possess antigenic factors which indicate them to be of host origin (*Sparck* 1962 1969)

The intention of the present work was to try by histological technique to elucidate further the role of the mesenchyme in tumour development By studying the morphology of the connective tissue reactions released at different times by different tumour transplants it should be possible to obtain a better understanding of the relation of these reactions to the progressive growth of the tumour By this means the extent to which the growth is based on the proliferation of the transplanted cells or on the activation and proliferation of the host tissues could be estimated

MATERIALS AND METHODS

Mouse strains The mice used were of the inbred C_3H/Ssc and DBA/2 Ssc lines of the Statens Seruminstitut As recipients of tumour transplants only C_3H mice were used The tumour donors were either syngeneic C_3H mice or allogeneic DBA/2 mice

Tumour transplantation The tumours selected for transplantation were primary spontaneous mammary carcinomas About one month after the first observation the tumour was cut out and removed sterilely from its site under the skin The tumour tissue was cut into small fragments with a pair of scissors For the trans plantation one half of the tumour material was prepared as a suspension in Gey s solution of tissue fragments about 0 2 mm in diameter The other half of the tumour was made into a single cell suspension in Gey s solution by forcing tissue suspension in and out of a syringe The cell number in the suspension was counted by means of a hemocytometer and adjusted to 7×10^7 nucleated cells/ml

Seventy two C_3H mice (4 months of age about 25 g) were divided into four groups of 18 (in each group half male half female) and each animal was given an injection subcutaneously in the right flank of one of the tumour preparations Group I was given 0 1 ml of C H cell suspension group II 0 1 ml of C H fragment suspension group III 0 1 ml DBA cell suspension group IV 0 1 ml DBA fragment suspension

Histology At the following 18 times after injection one animal of each group was killed 1 hour 4 hours 12 hours 1 day 2 days and so on until 14 days after injection By means of a dye the transplantation site was marked in each recipient The area of the implant including skin and subcutaneous tissues was cut out from one animal per group at the above mentioned times after grafting The material was fixed in Davidson s solution and was thereafter embedded in paraffin and prepared in the usual way for histological sections The sections were stained with haematoxylin eosin or with special staining methods for connective tissue elements such as van Gieson and Mallory

HISTOLOGICAL FINDINGS

When the preparations were examined microscopically it was found that the implanted tissue was destroyed so rapidly in the case of both antigenic and non antigenic transplants that it was impossible already

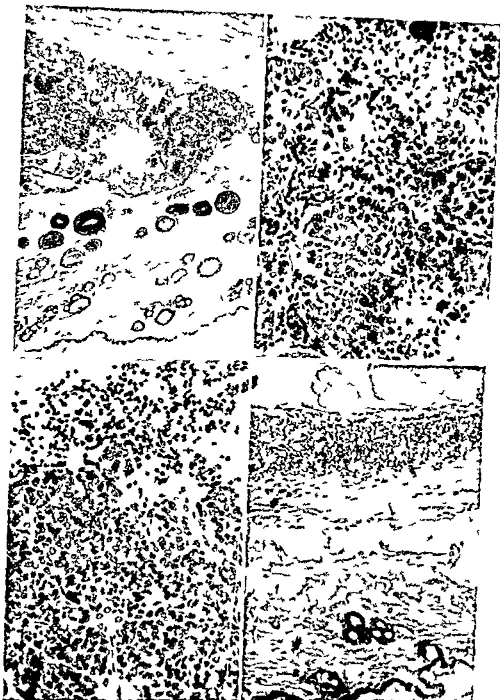


Fig 1 Allogeneic graft 1 hr (× 70)
Fig 2 Allogeneic graft 1 hr (× 300)
Fig 3 Syngeneic graft 1 hr (× 300)
Fig 4 Syngeneic graft 8 hrs (× 150)



Fig Syngeneic graft 8 hrs (× 600)
Fig 6 Allogeneic graft 8 hrs (× 300)
Fig 7 Syngeneic graft 12 hrs (× 150)
Fig 8 Syngeneic graft 12 hrs (× 300)

after a few hours to distinguish histologically between the transplants of cell suspension and those of fragments Therefore the mice that received suspensions and fragments are dealt with as one group in the following

However the microscopical examination of tissue samples disclosed that after the lapse of about 3 days characteristic differences appeared between the host connective tissue reactions in compatible (syngeneic) and incompatible (allogeneic) donor host combinations Figs 1–24 show successive stages of the development in the transplant and surrounding tissues during the first 9 days after grafting in the syngeneic and the allogeneic combinations All the sections shown are stained with haematoxylin eosin Fig 1 shows the site of the inoculated tumour tissue 1 hour after inoculation in the subcorial loose connective tissue In this case the donor is of the allogeneic strain In Fig 2 it can be seen that the tumour tissue is composed not only of carcinomal structures but also of round cell elements of reactive infiltration From the following figure (Fig 3) showing a syngeneic tumour graft 1 hour after implantation it is seen how in this case also the tumour tissue is composed of adenoid carcinomal structures and round cell elements

Already after the lapse of 4 hours a marked destruction of the cells of the tumour implant can be observed in both tumour donor combinations a process which is still more pronounced after 8 hours This can be seen from the following three figures Fig 4 is a section of the skin with a syngeneic tumour graft in the subcutaneous muscular layer With higher magnification (Fig 5) it can be seen that already after 8 hours the implanted material is mostly destroyed dominated by cell debris and infiltrated with polymorphonuclear leucocytes In the next figure (Fig 6) showing an allogeneic tumour graft 8 hours after inoculation the microscopical picture is the same and only a few clumps of tumour cells are still preserved

After 12 hours the process of necrosis is still more pronounced with only a few degenerating tumour cells as shown in Fig 7 from the syngeneic transplantation Although the picture is very similar to that after 8 hours here the beginning response in the surroundings can be seen in the form of extreme dilations of vessels some activation of the cells of the loose connective tissue and a marked hyperplasia of the epidermis overlaying the graft Fig 8 with higher magnification shows the proliferation of the cells in the epidermis Fig 9 shows that also in the allogeneic tumour transplantation after 12 hours a few degenerating residual tumour elements are left with inflammatory reaction of the surrounding connective tissue The main part of the graft however is already destroyed and forms a necrotic debris

Fig 10 shows a section of the transplantation site after the lapse of one day after syngeneic grafting It can be seen that the process of destruction is continuing but that still a few clumps and single tumour cells can be found At that time (after one day) there is not yet any



Fig 9 Allogeneic graft 12 hrs (× 300)
Fig 10 Syngeneic graft 1 day (× 300)
Fig 11 Allogeneic graft 2 days (× 300)
Fig 12 Syngeneic graft 3 days (× 50)

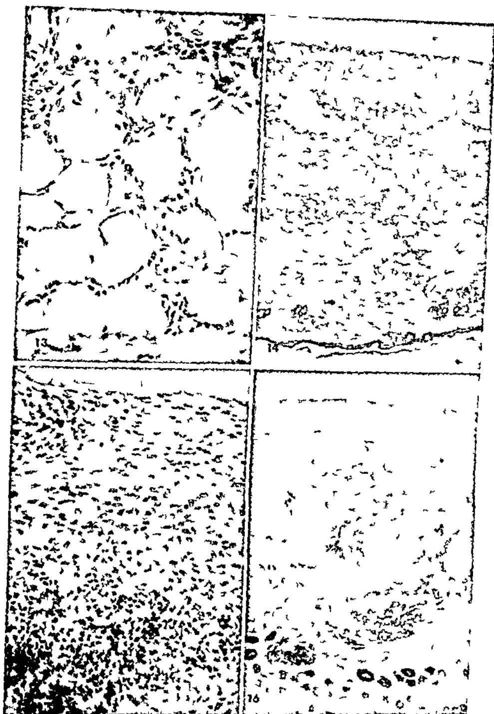


Fig 13 Syngeneic graft 3 days (× 300)
Fig 14 Allogeneic graft 3days (× 70)
Fig 15 Allogeneic graft 3 days (× 300)
Fig 16 Syngeneic graft 4 days (× 50)

obvious difference between the syngeneic and the allogeneic transplantation

However on the second day in addition to the destructive features there are signs of activation of the endothelial cells in the dilated vessels and of the mesenchymal cells of the host connective tissue especially in the case of an *allogeneic donor* (see Fig 11) At that stage this is not yet quite so apparent in the syngeneic combination

Already on the third day there is a clear difference in the mode of reaction of the host connective tissue in the two types of tumour transplantation In both cases there is activation of the mesenchymal cells of the connective tissue *around* the necrotic graft This reaction seems generally to be more intense in the case of the antigenic graft than with a non antigenic syngeneic graft In the latter case however (Fig 12) the activation of the mesenchymal cells of the connective tissue is furthermore characterized by the fact that in certain places mainly around the obstructed capillaries the activated cells engage in a proliferative process Microscopically (Fig 13) these cells distinguish themselves by polymorphy of the nuclei and by a more pronounced staining As can be seen from the section shown in Figs 12 and 13 it seems quite obvious that this proliferation is initiated in the *surroundings* rather than within the implant The next two figures (Figs 14 and 15) show in survey and in detail how in the antigenic non compatible combination the mesenchymal reaction around the necrotic graft is intense though without proliferative features

The following two figures show the histological picture after the lapse of 4 days In the syngeneic combination (Fig 16) the proliferation of cells in different places of the surrounding host connective tissue can be seen The implanted tumour tissue is necrotic throughout In the allogeneic combination (Fig 17) an activation of the connective tissue is seen but this is of a different character without any proliferative foci

Fig 18 (from the 5th day) shows in a higher magnification how in the compatible syngeneic tumour donor combination the proliferation typically begins around small obstructed capillaries From the histological appearance it does not seem unlikely that these proliferating cells are endothelial or pericapillary cells In the incompatible allogeneic combination (Fig 19) the reactive connective tissue is infiltrated with inflammatory cells and has the appearance of a young granulation tissue

During the following days the two different processes progress further On the 6th day it can be seen how in the syngeneic combination (Fig 20) the tumour has developed from multicentric foci and how the early tumorous structures are in continuous connection with the host connective tissue The next figure (Fig 21) shows the tissue reaction on the 6th day after an allogeneic graft with a microscopical picture of a young granulation tissue

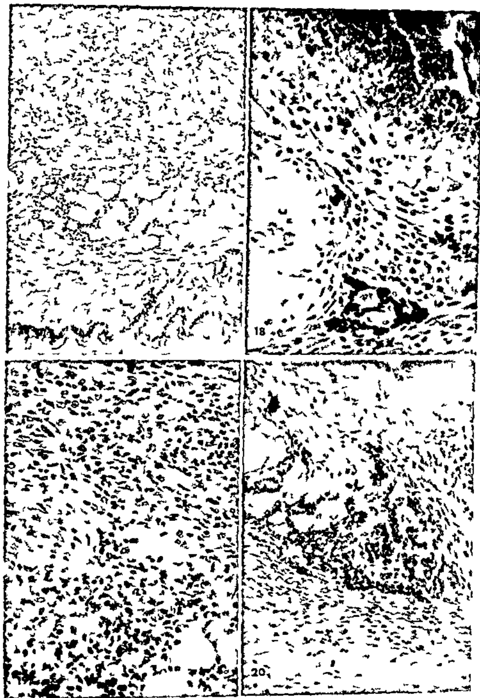


Fig 17 Allogeneic graft 4 days (× 100)
Fig 18 Syngeneic graft 5 days (× 300)
Fig 19 Allogeneic graft 5 days (× 300)
Fig 20 Syngeneic graft 6 days (× 140)

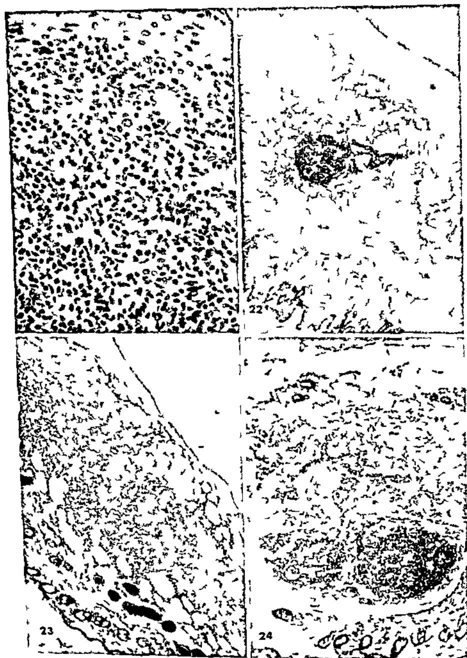


Fig 21 Allogeneic graft 6 days (× 300)
Fig 22 Syngeneic graft 7 days (× 50)
Fig 23 Allogeneic graft 7 days (× 70)
Fig 24 Syngeneic graft 9 days (× 70)

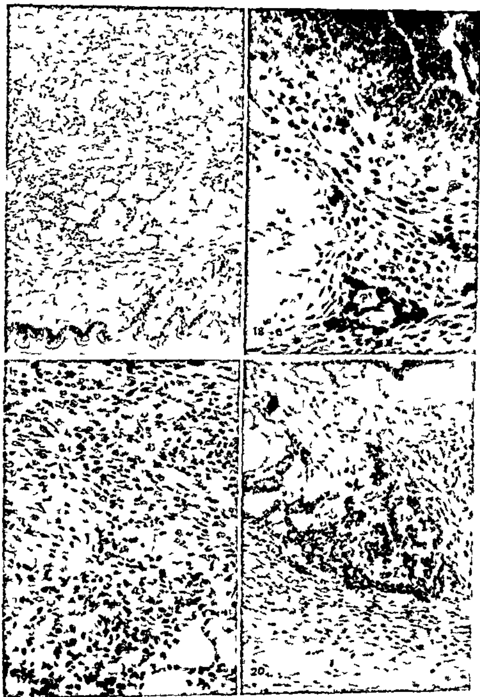


Fig 17 Allogeneic graft 4 days (× 100)
Fig 18 Syngeneic graft 5 days (× 300)
Fig 19 Allogeneic graft 5 days (× 300)
Fig 20 Syngeneic graft 6 days (× 140)

a hindrance to continuous growth by stimulating an acute non proliferative inflammation

Numerous findings are reported which suggest that the growth of tumours may depend on host connective tissue proliferation Available reports show that increased inflammatory activity at the site of tumour implants results in enhanced tumour growth (*Jones & Rous* 1914 *Kubo* 1930 *Zahl & Nowak* 1949 *Selye* 1957 *Vasiliev* 1958) It was shown by *Vasiliev & Olshevskaja* (1958) that the simultaneous injection of embryonic tissue together with the tumour resulted in an increased tumour growth accompanied by an accumulation in the region of large numbers of immature fibroblasts Such findings indicate that an active host connective tissue response is essential for the establishment and outgrowth of the transplantation tumour at least by providing a vascular supply and a supporting stroma Also viral oncogenesis has been shown to depend on an interaction with the mesenchyme Infection of rudiments of mouse salivary glands with polyoma virus gave rise to tumours only when mesenchymal and epithelial components of the organ were combined (*Dawe Morgan & Slatick* 1966)

There are reports which show accordingly that the suppression of connective tissue proliferation and initial inflammation results in an inhibited tumour growth Thus cortisone may inhibit the growth of transplanted tumours in certain donor host combinations (*Antopol Glaubach & Graff* 1954 *Baserga & Shubik* 1954 *Vasiliev* 1958 *Sparck* 1962 1969) Correspondingly the suppression of host lympho reticular function by whole body X irradiation has been shown to inhibit the growth of transplanted murine carcinoma provided that no disparity existed between donor and host with respect to tissue antigens (*Sparck* 1961 1962 1969)

Wheatley and co workers also investigated the relationship between host response and tumour growth (*Wheatley & Ambrose* 1964 *Wheatley & Easty* 1964) They studied the invasive growth of transplanted mouse ascites tumour into the peritoneum and found that infiltration of the peritoneum by host leucocytes always precedes invasion The more intense this response of the host for instance through stimulation with irritants the more rapid the tumour development On the other hand suppression of host immune responsiveness by treatment with cortisone whole body irradiation or thymectomy reduced tumour development

All the findings referred to above and the histological observations reported in this paper agree in demonstrating the dependence of the tumour growth on the activity and proliferation of the host mesenchyme This is also in agreement with earlier studies on precancerous conditions of the cervix uteri (*Gross* 1964) It was shown that the cervical lesions are not based on a change of epithelial elements but start from the so called subcylindrical cells which are activated primitive mesenchymal cells The metaplasia passes through stages of local

hyperplasia with round cell infiltration such as those found in inflammatory reactions

Direct support to the conception of tumourogenesis as an activation of mesenchymal cells comes from experiments which on the basis of immunological criteria show that the tumour which develops after transplantation contains cells of host origin (*Sparck* 1962 1969) By using recipient mice which were F_1 hybrids between two inbred lines of mice (or which were chimaeras between them) and a tumour donor from one of the parental strains *an experimental system was obtained* in which the graft was compatible to the recipient and non rejectable However in such a system cells of donor and host origin could be distinguished by means of antigenic differences and a considerable contribution of the host tissue to the tumour could be established

On the whole the data *available seem to suggest the hypothesis* that the tumour is the result of an activation of the primitive mesenchymal cells of he host which are being transformed to tumour cells This conception would be in contrast to the germ layer theory but would be consistent with modern opinions attributing important functions *to the mesenchyme in regenerative and proliferative processes* The germ layer theory has been regarded as a fundamental law in biology for more than a century but evidence has been produced showing that the germ layers are not irreversibly determined in early development The classical work of *Spemann* and his school on transplantation *in amphibian embryos (for survey* see *Barth* 1953*) has shown clearly* that ectodermal structures can be converted into mesoderm and *vice versa* More recent studies on cell transformation *in vivo* (*Rebuck & Crowley* 1955 *Algower* 1956) and *in vitro* (*Pulvertaft & Jayne* 1953 *Berman & Stulberg* 1958) have demonstrated a considerable multi*potentiality of primitive reticular* and lymphoid cellular elements enabling such cells to transform not only to the cell types of the supporting connective tissue and a variety of blood cells but even to cells of epithelial morphology

SUMMARY

A report is given of a histological study carried out with the purpose of investigating to what extent the tumour growth following transplantation is based on the proliferation of the transplanted cells or on the activation and *proliferation of the host* tissues A transplantation system consisting of inbred C3H mice as recipients of spontaneous mammary tumours from C3H or DBA mice was used Tissue samples from the transplantation area were taken at different times after grafting (1 hour to 2 weeks) and microscopical observations made concerning *the morphology of the connective* tissue reactions released in different tumour host combinations

This investigation disclosed that the changes occurring during the

first two days were similar in compatible (syngeneic) and incompatible (allogeneic) donor host combinations and consisted of the disintegration of the inoculated tumour tissue and a commencing activation of the host mesenchyme However about three days after grafting the reaction of the host was characterized in the case of a syngeneic tumour donor by a further proliferation of the cells of the loose connective tissue (RES) accompanied by a tumorous development In contrast in the case of an allogeneic tumour donor the response of the host connective tissue did not take the same proliferative course but had the features of a subacute inflammation process with intense round cell infiltration leading to the formation of an ordinary granulation tissue

These observations support the view that the development of a tumour is not necessarily based on the autonomous proliferation of a tumour cell variant but may be the result of activation of primitive mesenchymal cells which contribute directly to the tumour tissue

REFERENCES

Algower M The cellular basis of wound repair Charles C Thomas Springfield 1956

Antopol W Glaubach S & Graff S Retardation of growth of implants of carcinoma 755 in cortisone injected mice Proc Soc exp Biol (N Y) *86* 364–366 1954

Barth L G Embryology 2 ed The Dryden Press N Y 1953 p 130–140

Baserga R & Shubik P The action of cortisone on transplanted and induced tumours in mice Cancer Res *14* 12–16 1954

Berman L & Stulberg C S The Detroit train of human epithelial like cells from nonleukemic peripheral blood Blood *13* 1149–1167 1958

Dawe C J Morgan W D & Slatick M S Influence of epithelio mesenchymal interactions on tumour induction by polyoma virus Int J Cancer *1* 419–450 1966

Gross K New experiences on the precancerous conditions of the cervix uteri Czechoslov Gynaecology *18* 403 412 1953

Gross K Studies on the mechanism of carcinogenesis Oncologia *18* 178–194 1964

Jones F S & Rous P On the cause of the localization of secondary tumours at points of injury J exp Med *20* 404–412 1914

Kubo H Experimentelle Untersuchungen uber das Verhalten zwischen Entzundung und Wachstum der Impfgeschwulste *7* Krebsforsch *31* 105–124 1930

Pulvertaft J V & Jayne W H W Agar culture of exudates Ann roy Coll Surg Engl *12* 161–173 1953

Rebuck J W & Crowley J H A method of studying leukocytic functions *in vivo* Ann N Y Acad Sci *59* 757–805 1955

Selye H Effect of inflammation upon the growth of transplantable neoplasms as demonstrated by the double granuloma pouch technique Brit J Cancer *11* 550–553 1957

Sparck J V Inhibition of transplants of spontaneous isologous tumours in mouse radiation chimaeras Acta path microbiol scand 51 403–415 1961

Spärck J V Immunity and host response in the growth of transplanted tumours Munksgaard Copenhagen 1962

Sparck J V The function of the immune response in the tumour growth and its genetical regulation Acta path microbiol scand 77 1–23 1969

Vasiliev J M The role of connective tissue proliferation in invasive growth of normal and malignant tissues A review Brit J Cancer *12* 524–536 1958

Vasiliev J M & Olshevskaya I V Embryonic tissue as a stimulant of tumour homotransplant growth (In Russian with an English summary) Voprosy Onkologii 4 548–552 1958
Wheatley D N & Ambrose E J Tumour cell invasion from transplantable ascites tumours into host tissues Brit J Cancer *18* 730–742 1964
Wheatley D N & Easty G C The growth and infiltration of Ehrlich s ascites tumour in mice with reduced immunological responses Brit J Cancer *18* 743–755 1964
Zahl P A & Nowak A Effect of subcutaneous injury on tumour growth in the mouse Proc Soc exp Biol (N Y) *70* 266–272 1949

The University Institute for Experimental Medicine (Head Jens Hing M D)
71 Norre Alle Copenhagen 2100 O Denmark.

RATE AND WAYS OF RESOLUTION OF HYPERTENSIVE VASCULAR DISEASE

By

FINN OLSEN

Received 26 II 69

Fibrinoid arteriolonecrosis developed in animals with experimental renal hypertension is a reversible condition which disappears a variable time after the return of the blood pressure to the normal level The healing takes place without conspicuous injury or with some cellulofibrous intimal and/or medial thickening (*Allison et al* 1967 *Kojimahara* 1967) Rats with renal hypertension react with a characteristic pattern of arterioles showing dilatations and constrictions along their courses (*Byrom* 1954) Such pattern can also be caused by intravenous injections of angiotensin (*Giese* 1964) and dilatations developed in this way are sometimes permeable for circulating colloidal carbon particles and fluorescent proteins (*Giese* 1964 *Olsen* 1968) A penetration of plasma components into walls of dilated arterioles seems to be correlated to a destruction of the internal elastic membrane (*Olsen* 1969)

The aim of the present work has been to examine the rate and route of elimination of colloidal carbon particles and fluorescent proteins deposited in the walls of arterioles in the mesentery and the submucosa of the small intestine after an exactly defined period of hypertension and the rate of regeneration of destructions of the internal elastic membrane after penetration of plasma components into the arteriolar wall

MATERIAL AND METHODS

Animals White female rats weighing 180-200 grams were used

Anaesthesia A solution of pentymal sodium 25 mg/ml was injected intraperitoneally at the rate of ½ ml per 100 grams of rat

Fluorescent proteins were produced as described in a previous paper (*Olsen* 1968) and their location in the walls of the arterioles studied under a fluorescent microscope

Colloidal carbon particles specially produced for experimental use (Gunther Wagner Pelikan Werke Hannover Germany C 11/1431 a)

Angiotensin II amid Hypertensin CIBA was dissolved in physiological saline

The experimental technique *Group 1* After complete anaesthesia had been in

This work was supported by grants from *Kong Christian X s fond* and the grant of Ebba Celinder

duced a catheter of polyethylene was placed in both femoral veins of ten rats *About 85 mg of Lissamine Rhodamine R B 200 labelled homologous serum proteins* were injected intravenously in the course of two minutes Ten minutes later ½–1 microgram of angiotensin was injected intravenously through the other catheter every 5th minute during 3 hours At the end of the experiments *0 1 ml of colloidal* carbon particles was injected intravenously The rats were killed by a large dose of pentymal sodium injected intraperitoneally 1–10 days after the experiment were terminated The small intestine was fixed in a 10 per cent *formalin solution* After dehydration in alcohol and clearing in methyl benzoate arterioles in the mesentery and the submucosa *of the small intestine with deposition of colloidal* carbon particles in the wall were dissected out under a stereo microscope The arterioles were embedded in paraffin cut in five micron thick serial sections and examined under the fluorescent microscope Finally the sections were stained with periodic acid—Schiff's reagent (PAS) or with orcein or with a combination of the two stains

Group 2 In six experiments the regional lymph nodes of the intestine and the peripheral blood were examined for cells containing phagocytized colloidal carbon particles in the cytoplasm 1–5 days after colloidal carbon particles had deposited in the arteriolar walls in rats with intermittent angiotensin hypertension with a duration of three hours

Group 3 Twelve controls received intravenous injection of 0 1 ml of colloidal carbon particles The regional lymph nodes of the intestine and the peripheral blood from these rats were examined for cells with phagocytized colloidal carbon particles in the cytoplasm hours and days after the injection of the colloidal carbon particles

TABLE 1

Days of survival of *rats after termination* of the experimental period	Examined arterioles *in which carbon* particles were deposited in the wall	*Number of arterioles* with deposition of *carbon which also* contained fluorescent proteins	Number of arterioles with deposition of *carbon and fluorescent proteins* which showed necrosis of the wall
1	13	12	2
2	11	10	2
3	11	3	0
4	9	0	0
5	10	0	0
6	12	0	0
7	9	1	0
8	9	0	0
9	8	0	0
10	9	0	0
13	4	0	0

RESULTS

Group 1 Elimination of colloidal carbon particles and fluorescent homologous serum proteins from the arteriolar walls

The results of these examinations are seen in Table 1 which shows that deposition of colloidal carbon particles in the arteriolar walls has been found 13 days after the deposition of the carbon took place At that time the deposition of the carbon appeared as a few particles so that it can be concluded that the rate of elimination of colloidal carbon particles from the arteriolar walls was about 13–15 days The rate of elimination of fluorescent proteins was 2–3 days with the ex



Fig 1

Part of an arteriole with a small deposit of colloidal carbon particles in the media Two mononuclear cells situated in the adventitia have phagocytized carbon particles (arrows) Magnification 2000 ×

ception of one case in which a grain of fluorescent protein in the adventitia in three consecutive sections of an arteriole was found seven days after the fluorescent proteins had deposited in the wall PAS positivity and disappearance of nuclei in the smooth muscle cells in the media was found in four arterioles The location of the medial necrosis corresponded to the deposition of the fluorescent proteins in the arteriolar wall

Concerning the elimination of the colloidal carbon particles from the arteriolar walls it was seen by stereo microscopic examination of the fixed and cleared loop of the small intestine that it took place as a transport of the colloidal carbon particles from the arteriolar wall to the connective tissue surrounding the arteriole This transport seemed to be very active 2–3 days after the deposition in the arteriolar wall had taken place Examination of serial sections after PAS staining showed that mononuclear cells had flocked to that part of the arteriolar wall in which colloidal carbon particles and fluorescent proteins were deposited and that the colloidal carbon particles situated in the connective tissue surrounding the arteriole were phagocytized by mononuclear cells (Fig 1) and transported away by the lymph This finding was observed in about every second serial section In some sections it was found that mononuclear cells apparently had penetrated into the arteriolar wall and phagocytized colloidal carbon particles deposited in the wall (Fig 2)

Besides the transport of the colloidal carbon particles from the wall of the arteriole to the surrounding connective tissue it was found that the elimination possibly also took place as a transport in luminal direction as colloidal carbon particles often were seen to be situated



Fig 2

Part of an arteriole It is supposed that mononuclear cells have penetrated into the arteriolar wall One of the cells (arrow) has phagocytized colloidal carbon particles Furthermore four leucocytes are seen in relation to the endothelium Two of these have phagocytized colloidal carbon particles Magnification 2000 ×



Fig 3

Part of an arteriole with a large deposit of colloidal carbon particles in the wall A leucocyte which seems to be mononuclear is situated in close relation to the endothelium corresponding to the deposition of the carbon in the wall The leucocyte has phagocytized colloidal carbon particles Magnification 2000 × Inserted in *the right upper corner a lymphocyte from the peripheral blood with phagocytized* colloidal carbon particles in the cytoplasm Magnification 1800 × The leucocyte *situated in the lumen of the arteriole in close relation to the endothelium has* shrunk in relation to the fixation while this has not been the case with the inserted blood lymphocyte

partly in the intima and partly in endothelial cells and in leucocytes *in the lumen of the arteriole* The leucocytes were situated in close relation to the endothelial cells corresponding to the deposition of the colloidal carbon particles in the arteriolar wall In the formalin fixed preparation it was difficult to determine the type of leucocytes to which the cells belonged but they seemed to be mononuclear (Fig 3)



A B

Fig 4

A Part of an arteriol with deposition of fluorescent proteins in the wall Accumulations of fluorescent proteins (arrow) in the connective tissue surrounding the arteriole B PAS staining of the section in Fig 4A Some of the mononuclear cells in the connective tissue are situated exactly corresponding to the situation of the fluorescent proteins Magnification 2000 ×



A B

Fig 5

A Part of an arteriole demonstrating a grain of fluorescent protein (arrow) in the lumen of the arteriole in close relation to the endothelium B PAS staining of the section in Fig 5A A leucocyte (arrow) which seems to be mononuclear is seen situated in close relation to the endothelium exactly corresponding to the situation of the grain of fluorescent protein Magnification 2000 ×

Concerning the elimination of the fluorescent proteins it was often seen that accumulations were situated in the connective tissue surrounding an arteriole with deposition of fluorescent proteins in the wall (Fig 4A) Under the fluorescent microscope it was impossible to determine whether the fluorescent proteins were situated in mononuclear cells but after PAS staining accumulations of mononuclear cells were seen in the connective tissue exactly corresponding to the location of the fluorescent proteins (Fig 4B) Like the elimination of colloidal carbon particles the elimination of the fluorescent proteins possibly also took place in luminal direction In a few cases a grain of fluorescent protein was found to be situated in the lumen of the arteriole in close relation to the endothelium and corresponding to a deposition of fluorescent proteins in the arteriolar wall (Fig 5A)

When stained with PAS a leucocyte which seemed to be mononuclear was seen to be situated close to the endothelium exactly corresponding to the situation of the grain of fluorescent protein (Fig 5B)

Regeneration of the internal elastic membrane In rats killed 1 3 6 8 and 10 days after the injection of angiotensin the healing of the internal elastic membrane was examined in arterioles in which colloidal carbon particles were deposited in the wall The results are seen in Table 2

TABLE 2

Days of survival of rats after the arteriolar deposition of carbon particles	Arterioles with *complete healing of the internal* elastic membrane	Arterioles with destroyed internal elastic membrane	Percentual healing of the internal elastic membrane in the arterioles
1	3	10	23
3	4	10	23
6	4	8	33
8	6	6	50
10	10	1	91

It is seen that 10 days after deposition of the colloidal c ticles the internal elastic membrane was healed in abo of the examined arterioles In some sections the membrane was found as a double outline correspondi but in most sections the continuity of the mem without visible defects

Group 2 (Examination of lymph nodes and in angiotensin treated rats) In the six experim gional lymph nodes of the intestine and the p examined for cells containing phagocytized c the lymph nodes were found to contain a few mo phagocytized colloidal carbon particles in the after the arteriolar deposition of colloidal carb was not possible to find such cells one day a the peripheral blood many small and large ly cytized colloidal carbon particles in the cytop 1–6 days after the deposition The number of cir with phagocytized carbon is seen in Table 3A

Group 3 (Examination of lymph nodes and th controls) The control experiments were p ods and with two different consignments of c Throughout the two periods examination of th of the intestine using the two different c icles failed to unveil any mononuclear cells w in the lymph nodes 1–5 days after the in ot particles In the first control period in which

of colloidal carbon particles was used it was not either possible to find lymphocytes with phagocytized colloidal carbon particles in the cytoplasm in the peripheral blood one and two hours and two and three days after the intravenous injection of colloidal carbon particles But in the second control period in which the second consignment of colloidal carbon particles was used a few *lymphocytes in the peripheral blood* were found to contain phagocytized colloidal carbon particles hours and days after the intravenous injection of these Table 3B shows the number of circulating lymphocytes with phagocytized carbon observed in the control experiments

TABLE 3A — TABLE 3B

Days after carbon particles had deposited in arteriolar walls	Number per thousand of lymphocytes in the peripheral blood having phagocytized carbon particles	Hours (h) or days (d) after the injection of carbon particles Control experiments	Number per thousand of lymphocytes in the peripheral blood having phagocytized carbon particles
1	30	2 h	1-2
2	22	4 h	1-2
3	24	6 h	2-3
4	21	1 d	2-3
5	21	2 d	2-3
6	19	4 d	2-3
		5 d	1-2

A comparison of the results recorded in tables 3A and 3B shows that the number of circulating lymphocytes with phagocytized colloidal carbon particles in the cytoplasm is about ten times higher in the experiments in which they were deposited in the arteriolar walls than numbers observed in the control experiments

In two control experiments 0 1 ml of colloidal carbon particles was injected intravenously followed by intravenous injections repeated during ten minutes of the same doses of angiotensin as these used in the experiments in groups 1 and 2 The aim of these control experiments was to see if angiotensin influenced the phagocytosis of the colloidal carbon particles of the blood lymphocytes Such an influence was not found as the number of lymphocytes with phagocytized colloidal carbon particles in the cytoplasm was identical with the values mentioned in table 3B corresponding to the first hours after the injection

DISCUSSION

The present results show that the rate of elimination of colloidal carbon particles deposited in the walls of arterioles damaged by intravenous injections of angiotensin is about 14 days The elimination of

similarly deposited fluorescent homologous serum proteins takes place in the course of a few days after the hypertensive influence on the arterioles has terminated This is in accordance with the finding that fibrinoid necrosis of the walls of arterioles was not manifest three weeks after the removal of the renal artery clamp in spite of the fact that such a necrosis was found before the removal of the clamp (*Allison et al* 1967)

Concerning *the route of elimination of the deposited colloidal carbon particles* it was evident that the carbon particles were transported from the vessel wall to the connective tissue surrounding the arteriole In the connective tissue the carbon particles were phagocytized by *mononuclear cells* However the question remains how this transport took place? Sometimes mononuclear cells apparently were found to phagocytize colloidal carbon particles in the arteriolar walls but it cannot be ruled out that cells in the arteriolar wall were able to phago cytize the carbon particles and transport these to the connective tissue *where they were phagocytized by histiocytes* The latter route of elim ination would be in accordance with findings in an electron microscopic study on the fate of histamine induced intramural vascular deposits of colloidal carbon particles in venules (*Cotran et al* 1965) which were phagocytized by endothelial cells and pericytes The slow out *ward transfer* of carbon particles was probably due to outward regur gitation of carbon by pericytes and finally to phagocytosis of the regurgitated carbon by histiocytes situated in the connective tissue around the venule

Besides the transport of the deposited colloidal carbon particles from the arteriolar wall to the connective tissue surrounding the arteriole it was found in the present work that a similar transport possibly also took place in luminal direction as colloidal carbon par ticles were found to be phagocytized by lymphocytes situated in the lumen of the arteriole in close relation to the depositions in the arteriolar wall By way of a comparison of the number of *blood lym phocytes* with phagocytized colloidal carbon particles in the cyto plasm observed in the experiments in which carbon particles were deposited in the arteriolar walls and the number of lymphocytes containing carbon particles in the cytoplasm observed in the control experiments it was found that there was a *difference about ten times* between these two groups It is therefore concluded that most of the phagocytized colloidal carbon particles in the lymphocytes in angio tensin treated rats arise from the deposits in the arteriolar walls *In* a paper on production and resolution of hypertensive lesions (*Allison et al* (1967) described that one *rabbit showed intense and extensive* arteriolar lesions with intimal and medial cellular necrosis and exudate of fibrin and protein Leucocytes adhered to the endothelial surface but no real thrombi were present This observation is perhaps *identical* with the finding in the present work namely that *lymphocytes* were

in close relation to the endothelium with phagocytized colloidal carbon particles in the cytoplasm

The *elimination of fluorescent proteins* seemed also to take place as a transport from the arteriole both to the connective tissue and into the lumen Whether a decomposition of the fluorescent proteins took place inside the wall of the arteriole as shown in an electron and light microscopic study on the elimination of intimal fibrinoid necrosis in arterioles (*Kojimahara* 1967) is so far unknown

In a previous paper (*Olsen* 1969) it was reported that a penetration of colloidal carbon particles into arteriolar walls was correlated to a destruction of the internal elastic membrane the term destruction meaning any discontinuation of the membrane small as well as large which was found in the immediately frozen arterioles This again means that while some of the destructions were marked the highest degree being absence of the membrane corresponding to about ¼ of the circumference of the arteriole other socalled destructions could be the morphological appearance of distensions of the fenestrae in the internal elastic membrane found by electron microscopy of normal arterioles (*Moore & Ruska* 1957 *Movat & Fernando* 1963)

While the previous studies (1969) of the morphology of the internal elastic membrane were performed on dilatations which were fixed by freezing the present study on the *regeneration of the internal elastic membrane* used formalin fixation by which the dilatations are found to collapse This change in method of fixation may possibly be the cause why lack of discontinuation of the membrane was found in about 20 per cent of the arterioles 24 hours after the deposition of the carbon particles if these cases represent discontinuations due to distended fenestrae and not real destructions The remaining about 80 per cent of the dilatations which still showed discontinuations of the membrane in a collapsed condition 24 hours after the experiment was terminated are believed to represent true destructions the grade of which was found to range from about $^1/_4$ to about $^1/_{10}$ *of the cir*cumference of the arteriole The regeneration of these destructions in the membrane was followed until ten days after the experiment was *terminated* at which time a regeneration with a continuous membrane was found in about 90 per cent *of the arterioles*

SUMMARY

The rate of elimination of colloidal carbon particles and fluorescent homologous serum proteins deposited in *arteriolar walls* as a consequence of arterial hypertension after *intravenous* injections of angiotensin is 13–15 days and 2–3 days respectively after the hypertensive influence on the arterioles has *terminated*

The colloidal carbon particles and the fluorescent proteins were eliminated from the arteriolar walls by way of a transport to the

surrounding connective tissue where both were found to be phagocytized by mononuclear cells The elimination took possibly also place in luminal direction as colloidal carbon particles and fluorescent proteins were found in endothelial cells and in leucocytes in the lumen of the arteriole close to the endothelium and corresponding to the deposition in the wall of the arteriole

One day after deposition of colloidal carbon particles in arteriolar walls the internal elastic membrane was found to be continuous around the whole circumference in 23 per cent of the examined arterioles Ten days after the deposition 91 per cent of the arterioles showed a continuous internal elastic membrane

REFERENCES

1 *Allison P R Bleehan N Brown W Pickering G W Robb Smith A H T & Russell R P* The production and resolution of hypertensive vascular lesions in the rabbit Clinical science 33 39–51 1967

2 *Byrom F B* The pathogenesis of hypertensive encephalopathy and its relation to the malignant phase of hypertension Experimental evidence from the hypertensive rat Lancet II 201–211 1954

3 *Cotran R S Gattuta M L & Majno G* Studies on inflammation American journal of pathology 47 1045–1078 1965

4 *Giese J* Acute hypertensive vascular disease 2 Acta path microbiol scand 62 497–515 1964

5 *Kojimahara M* Healing and exacerbation of arterial lesions in rats with experimental hypertension with special reference to effects of antihypertensive drugs on arterial lesions The gumma journal of medical sciences 16 1 42 1967

6 *Moore D H & Ruska H* The fine structure of capillaries and small arteries Journal biophysic and biochem cytol 3 457–462 1957

7 *Movat H Z & Fernando N V P* The fine structure of the terminal vascular bed I Small arteries with an internal elastic lamina Experimental mol path 2 549–563 1963

8 *Olsen F* Penetration of circulating fluorescent proteins into walls of arterioles and venules in rats with intermittent acute angiotensin hypertension Acta path microbiol scand 74 325–332 1968

9 *Olsen F* Arteriolar permeability and destruction of elastic membrane in hypertension Acta path microbiol scand 75 527 536 1969

Acta path microbiol scand 77 49–56 1969

The Institute of Pathology University of Lund Lund Sweden

GROWTH OF ROUS HAMSTER SARCOMA ON THE CHORIO ALLANTOIC MEMBRANE

By

CARL GUSTAF AHLSTRÖM and LARS GÖRAN LINDBERG

Received 15 iii 69

It has been reported (1 7) that rat tumours induced by Rous sarcoma virus (RSV) can be transplanted serially on the chorio allantoic membrane (CAM) of embryonated eggs eliciting virus release and multiplication It is shown in the present investigation that synthesis of infectious RSV occurs a few days after the inoculation of Rous hamster sarcoma on CAM An attempt is being made to correlate the anatomical finding on the CAM to the synthesis of the virus

EXPERIMENTAL PROCEDURES

The Rous hamster sarcoma was induced in 1963 by SR RSV and has since then been passed serially in newborn hamsters Inoculation of viable cells from the passage tumours into young chicks has regularly induced tumours at the site of injection producing infectious RSV whereas no tumours have appeared in chicks injected with cell free extracts from the hamster sarcoma The hamster tumour has in the course of the transfers gradually assumed an anaplastic highly hemorrhagic character and kills the hamsters in 10–12 days For inoculation on the CAM the sarcoma was finely minced and suspended 1 5 in PBS with antibiotics The suspension was filtered through 4 layered gauze and 0 5 – 2 × 10[6] viable cells were inoculated onto the dropped CAM in the conventional way Nine day old embryonated eggs of RIF free chickens were used The membranes were usually harvested after 7 days some times earlier and on some occasions after 9 days For serial transplantation the CAM lesions obtained were finely minced and suspended 1 5 in PBS

Cell free extracts of the hamster sarcoma and of the CAM lesions were obtained by homogenizing the suspended material in an *Ultrathurrax homogenizer (type PH 18/2 24 000 rpm)* for 6 minutes in the cold the suspension was then centrifuged for 0 minutes at 2 000 rpm The supernatant was cautiously removed and recentrifuged for the same time and at the same speed The procedure was repeated yet again Sometimes the supernatant fluid was repeatedly centrifuged at 13 800 rpm (10 000 g) for 30 minutes at —3 C the supernatant being cautiously pipetted off between each centrifugation The final supernatant was used for inoculation

The presence of virus in the extract was tested by injecting 0 25–0 5 ml of the fluid into the wing webs of one to two weeks old RIF free chickens or by inoculating 0 5 ml on the CAM of 9 day old RIF free eggs

---

Requests for reprints should be addressed to Institute of Pathology University of Lund S-220 05 Lund 5 Sweden

The present investigation was supported by grants from the *Swedish Cancer Society* from *Leonie Deshayes fond* and from *John och Augusta Perssons stiftelse* for medicinsk forskning

The skilful technical assistance of Mrs *B Carlen* Mrs *I S Hedström* and Miss *B Nordstrom* is greatly appreciated.

CAM lesions were fixed in formol or in Bouin s fluid Stainings Haematoxylin eosin v Gieson Gridley Kernechtrot PAS and Colloidal iron For electron micro scopy small pieces were fixed in 1 per cent glutaraldehyde in phosphate buffer and postfixed either in 2 per cent OsO_4 chromate fixative or in 3 33 per cent OsO_4 s collidine buffer The specimens were embedded in Vestopal W sectioned on a LKB Ultrotome III and examined in a Zeiss EM9 electron microscope equipped with a condensor

RESULTS AND COMMENTS

Most membranes inoculated with 0 5–2 × 10^6 hamster sarcoma cells (CAM passage 1) showed 7 days later numerous isolated or confluent plaques sometimes diffusely scattered but often especially prominent along the margin of the dropped membrane Some plaques were white most were more or less hemorrhagic Hemorrhagic blebs were often seen and clots of blood were sometimes visible on the surface of the membranes After inoculation of a suspension of finely minced plaques onto new membranes (CAM passage 2) similar but not so numerous lesions were obtained When these plaques were inoculated onto new eggs (CAM passage 3) the CAM lesions obtained had a deviating ap pearance the membranes were considerably swollen and showed numerous greyish white plaques of the same character as CAM lesions induced by RSV This course of events is the same as described by *Harris et al* (1) after serial transplantation of Rous rat sarcoma on CAM

Histologically the CAM lesions in passage 1 were mainly composed of hamster sarcoma cells In the vicinity of hamster cell groups how ever single small ill defined foci of elongated slightly swollen fibro blasts suggestive of early RSV lesions were found and 9 days after the inoculation some lesions were composed of a mixed population of hamster sarcoma cells and cells with the appearance of chicken sar coma cells The lesions on CAM passage 2 were mainly composed of hamster sarcoma cells but some small isolated plaques had the micro scopical character of RSV lesions The CAM lesions in passage 3 were almost in toto composed of Rous chicken sarcoma cells Only single degenerated hamster cells were found

Cell free extracts from the membranes injected into the wing webs of RIF-free chicks induced rapidly growing Rous sarcomas at the site

---

Figs 1–3

Fig 1 Four hamster cells embraced by chicken fibroblast like cells with slender nuclei and elongated cytoplasm CAM four days after inoculation of R us hamster sarcoma cells *Htx—v Gieson* × 1 000

Fig 2 A group of hamster cells closely surrounded by flattened fibroblast like chicken cells CAM four days after inoculation of Rous hamster sarcoma cells Htx—v Gieson × 1 000

Fig 3 A necrotic hamster cell is seen in the centre of the figure surrounded by a chicken cell with a small oval nucleus and elongated cytoplasm CAM three days after the inoculation of hams er sarcoma cells Htx—v Gieson × 1 000


1
2
3



Fig 4
A large rounded Rous hamster sarcoma cell almost completely embraced by a slender mesodermal CAM cell. A small space between the cells can be discerned but several point to point contacts are seen. × 8 000

of injection and typical RSV plaques appeared on CAM's inoculated with the same material. This applied also to extracts from CAM passage I.

Assuming that a transmission of RSV genome from the hamster sarcoma cell to the chicken cell is necessary for the production of

infectious RSV a morphological analysis of the relationship between the transplanted hamster sarcoma cells and the CAM cells during passage 1 might be of interest The first day after inoculation the hamster sarcoma cells were mainly localized on the surface of the ectoderm but invaded in some areas the ectodermal layer The sarcoma cells could easily be distinguished by their size and their large slightly irregular nuclei with prominent nucleoli On the second day after inoculation small areas of the ectoderm were replaced by hamster cells The cells formed fringed protuberances above the level of the surrounding CAM but invaded also the underlying mesoderm Only very few lymphocytes and eosinophilic leukocytes appeared at this stage in the neighbourhood of the invading sarcoma cells A few days later the membrane showed solid masses of hamster sarcoma cells extending almost to the entoderm The sarcoma cells had often penetrated the vascular walls and many vessels were transformed to cysts filled with erythrocytes and tumour cells

It was possible during the first days after inoculation of the sarcoma cells to follow their invasion in some detail It was conspicuous that soon after the penetration of the ectoderm a close contact was established between the hamster cells and the mesodermal chicken cells One or several chicken fibroblasts were often glued to a single sarcoma cell and many hamster cells were seen to be embraced by mesodermal chicken cells (Fig 1) These embracing figures were seen also when the sarcoma cells were arranged in small groups each cell in the group being surrounded by a slender chicken fibroblast with a slightly swollen nucleus (Fig 2) The fibroblast nature of the embracing cells was as a rule evident but occasionally a capillary endothelial origin could not be excluded In some embraced hamster sarcoma cells the nucleus was only visible as a pale shadow indicating that the cell was dead (Fig 3) The embracing would then be equal to an engulfing process as in phagocytosis

It is reasonable to connect the close contacts between the invading hamster sarcoma cells and the chicken fibroblasts with the release of infectious RSV which is known to occur when viable Rous mammalian sarcoma cells are transplanted into a susceptible chicken It has recently been demonstrated by *Svoboda et al* (4 5) that the yield of infectious RSV from a mixed culture of chicken fibroblasts and Rous rat sarcoma cells is greatly increased by the agglutinating effect of Sendai virus and *Lindberg et al* (3) has presented electron microscopical findings indicating an intimate contact between co cultivated Rous mouse sarcoma cells and normal chicken fibroblasts

The ultrastructure of the chorio allantois of chicks at various times of incubation and the structure of RSV infected CAM have recently been described (2 6) The Rous hamster sarcoma cells can easily be distinguished from the CAM chicken cells by their much larger and almost spherical size by the numerous polyribosomes in the cytoplasm



Fig. 5

A chicken fibroblast with swollen and elongated nucleus and numerous densely packed endoplasmic membranes and mitochondria intimately encircling a hamster sarcoma cell with comparatively less ectoplasmic organelles. This suggests a further development of the process demonstrated in Fig. 4. × 6 500

and by the relatively sparse number of cytoplasmic organelles It offered no difficulties to recognize the embracing figures in the electron microscope Several hamster sarcoma cells were almost completely encircled by slender chicken fibroblasts (Fig 4) A narrow space between the two cells could be discerned but often this space was traversed by tiny cytoplasmic processes In other cells these point to point contacts were extended to broad zones (Fig 5) Immediately under the plasma membranes of two closely adjacent cells the cytoplasm occasionally showed a conspicuous absence of cell organelles and especially of polyribosomes This might indicate an impaired function of the membranes thus allowing a direct transfer of compounds between the two cells It is conceivable that a further progression of the intimate contact between the chicken and the hamster cell might end in complete phagocytosis

Four days after the inoculation of the sarcoma cells in CAM passage 1 extracellular virus particles of RSV type were observed as well as budding pictures from the membranes of chicken cells

The anatomical findings described above may offer a basis for a hypothetical transmission of RSV genome from the Rous hamster sarcoma cells to the chicken cells Further investigation along similar lines will be carried out on other types of Rous mammalian sarcomas

SUMMARY

A Rous virus induced hamster sarcoma was serially transplanted on CAM The lesions obtained were in the first two passages mainly composed of hamster sarcoma cells in passage three of chicken sarcoma cells Infectious RSV was found in all CAM passages and RSV lesions as well as Rous virus particles were seen already in CAM passage 1 A cell to cell contact could be demonstrated between the invading hamster sarcoma cells and the mesodermal chicken cells which might enable a transmission of RSV genome from the hamster sarcoma cell to the chicken cell

REFERENCES

1 *Harris R J C & Chesterman V B* Growth of Rous sarcoma in rats ferrets and hamsters NCI Monograph *17* 321-335 1964
2 *Leeson T S & Leeson C R* The chorio allantois of the chick Light and electron microscopic observations at various times of incubation J Anat *97* 585-595 1963
3 *Lindberg L G & Jonsson N* Types of contacts *in vitro* between Rous mouse tumour cells and chicken fibroblasts as studied with the electron microscope Acta path microbiol scand 73 359-368 1968
4 *Svoboda J Hložanek L & Machala O* Quantitative assay system for the transmission of RSV genome from virogenic mammalian cells into the chicken cell with the help of the Sendai virus Folia Biol (Praha) *14* 26-33 1968
5 *Svoboda J Machala O & Hložanek I* Influence of Sendai virus on RSV formation in mixed culture of virogenic mammalian cells and chicken fibroblasts Folia Biol (Praha) *13* 155 157 1967

6 *Sweeny P R & Bather R* An electron microscopic study of the chorioallantoic membrane following the infection with Rous sarcoma virus J Cell Biol 36 299–311 1968
7 *Vrba M & Chaylor D* An attempt to detect and titrate Rous sarcoma virus producing cells of rat tumour XC on the chorioallantoic membrane of the chick embryo Folio Biol (Praha) *10* 50–53 1964

Acta path microbiol scand 77 57 65 1969

Institute of Pathology University of Lund Lund Sweden

FURTHER STUDIES ON THE INTERACTION *IN VITRO* BETWEEN MAMMALIAN ROUS SARCOMA CELLS AND CHICKEN FIBROBLASTS

By

NILS JONSSON

Received 15 iv 69

The transformation of chicken embryonic fibroblasts (CEF) and initiation of RSV production by co cultivation of tumour cells from RSV-induced mammalian sarcomas and CEF is generally assumed to involve a transmission of the RSV genome from the mammalian sarcoma cell to the chicken cell without the participation of complete biologically active virus particles The mechanism of this transmission has been studied very intensely during the last few years (*Simkovic et al* 1964 *Svoboda* 1964 *Vigier & Svoboda* 1965 *Jonsson* 1966 *Lindberg & Jonsson* 1968) Three possibilities have been taken into consideration

Although most mammalian Rous tumours do not release any demonstrable infectious virus neither *in vitro* nor *in vivo* (for review see *Ahlstrom* 1964 *Svoboda et al* 1963 *Jonsson* 1966) evidence has been presented that some rodent cells are able to synthesize mature virus (*Svoboda* 1964) or at least viral antigen (*Shevlyaghin & Martirosyan* 1966) It thus cannot be excluded that the process of transmission involves the release of virus from the mammalian tumour cells in such small amounts or in such a labile state that it cannot be demonstrated by commonly used techniques A virus production by the rodent tumour cells after transfer of some hypothetical substance from the CEF must also be taken into consideration

Secondly the viral genome could be transferred to the chicken cells by the passage of some subviral determinant presumably by means of some type of cell to cell contact Support to this view has been given by *Svoboda et al* (1968) and the possible morphological basis of such contacts has been discussed by *Lindberg & Jonsson* (1968) *in vitro* and by *Ahlstrom & Lindberg* (1969) on the chorioallantoic membrane of embryonated eggs inoculated with Rous hamster sarcoma cells

A third possibility is the complete fusion of two types of cells with a formation of heterokaryons This mechanism would require a virus

---

This work was supported by grants from the *Swedish Cancer Society* and *Lennie Deshayes fond*

synthesis by these heterokaryons infecting new CEF with a formation of transformed foci unless fused cells could segregate again as the heterokaryons would soon be eleminated from the mixed cultures Evidence of this mechanism has been presented by *Vigier & Montagnier* (1966)

As it seemed of interest to study the possible mechanism attempts were made to initiate RSV production by means of co cultivating irradiated CEF and rodent Rous sarcoma cells as well as attempts to demonstrate viral antigen in rodent tumour cell preparations capable of absorbing antiviral activity from antiviral immune serum Furthermore attempts were made to interfere with the infection of CEF in mixed culture by means of antiviral immune serum that would be expected to inhibit infection via free virus particles

MATERIAL AND METHODS

Chick embryo cultures Primary cultures were prepared from 9-11 day old chick embryos in 100 mm petri dishes (Falcon) essentially according to the technique of *Temin & Rubin* (1958) Tests for viral activity were performed on secondary cultures prepared 4-5 days later Only embryos highly susceptible to SR RSV were used During the later part of the experiment embryos could be obtained from an isolated RIF free stock of white Leghorn (Vinterleds gård Läggesta Sweden) These embryos proved highly and almost uniformly susceptible to SR RSV

Virus The same SR RSV pool was used in all experiments It was prepared by using a simplified method of *Bryan et al* (1954) as described previously (*Jonsson & Sjögren* 1965) The titre was 2 × 10^4 FFU/ml

Antiviral immune sera against SR RSV were collected from chickens after an immunization procedure according to *Fink & Rauscher* (1961) using heat inactivated virus and subsequently repeated challenges with live virus Sera from individual birds or pooled sera were stored at —20 C The antiviral activity was assayed according to the technique of *Rubin et al* (1960 1961) After inactivation at 56 C for 30 minutes serum dilutions were incubated for 30 minutes at 37 C with the appropriate virus dilution A 90 per cent reduction of the number of foci was required for neutralization The highest serum dilution giving this neutralization was used in the absorption tests Control serum derived from untreated chickens

Tumour cells derived from sarcomas induced by sub utaneous inoculation of a suspension of SR RSV chicken sarcoma into newborn mice or rats The mouse tumours RSC RYC and RS57D induced in the inbred mouse strains A/Sn A BY and C57Bl/bl respectively have been used and described in previous papers (*Jonsson & Sjogren* 1965 *Jonsson* 1966) They all contain stable tumour specific transplantation antigens and have been repeatedly checked in mixed cultures for the presence of the RSV genome The tumour RRC690 was originally induced in a commercial white rat and has also been repeatedly tested for the presence of the viral genome All the tumours were kept in serial passage *in vitro* Tumour cell suspensions were prepared by trypsinization and the number of trypan blue unstained tumour cells was estimated (*Boyse et al* 1962)

Irradiation procedure Tumour cell suspensions were irradiated at 0 C by X rays generated at 200 kV 15 mA and filtered by 1 mm Al The dose was 8000 r

Disruption of the cells was performed by sonication (MSE ultrasonic power unit 18-20 kC/sec 10 × ½ min at 0 C) or by freezing and thawing repeated three times

Mixed cultures were prepared as described previously (*Jonsson* 1966) Known numbers of mammalian tumour cells were added to 1 2 × 10^6 CEF in 100 mm petri dishes In the antiserum experiments antiviral chicken immune serum or control chicken serum was added in the appropriate concentrations 4 6 days later cultures were passed to 60 mm petri dishes with the same antiserum concentrations in the medium and on the following day covered with agar medium The cultures were stained

with Giemsa 6 9 days later when virus infected control cultures showed evident foci As previously reported the system requires a certain number of tumour cells (about 5 × 10⁴ cells) to give registrable foci of transformed CFF and increase in this cell number results in a roughly linear increase of the number of foci

RESULTS

Effect of Irradiation or Sonication of the Chicken Fibroblasts

Tests for viral activity were performed on cell free media from cultures of Rous mouse tumour cells (5 × 10⁴ cells per culture) cultivated alone or after the addition of CEF untreated X irradiated or sonicated (2 × 10 cells per culture) The results of the tests performed at various ages of the cultures are collected in Table 1 It is apparent that virus synthesis can only be demonstrated in the presence of intact CEF and that virus synthesis can be induced neither in the mouse tumour cells after the (even repeated) addition of X ray damaged CEF or sonicated fragments of CEF nor in hypothetically formed heterokaryons from the mammalian tumour cells and irradiated CEF This is further confirmed by the negative finding in tests for viral activity in sonicated cell preparations from mixed cultures containing X irradiated or sonicated CLF

TABLE 1
Results of Tests for RSV Activity in the Media from Cultures of Mammalian Rous Sarcoma Cells (from the Three Mouse Tumours RS57D RSC and RYC) after the Addition of CEF Untreated X Irradiated or Sonicated

Type of culture	Amount of FFU/ml medium on day number			
	6–8	10–12	14 16	20–22
RS57D	0	0	n t	0
RS57D + CEF (untreated)	0	760	n t	360
RS57D + CEF (X irradiated)	0	0	n t	0
RS57D + CEF (sonicated)	0	0	n t	0
RSC	0	0	0	n t
RSC + CEF (untreated)	0	740	590	n t
RSC + CEF (X irradiated)	0	0	0	n t
RSC + CEF (sonicated)	0	0	0	n t
RYC	0	0	0	0
RYC + CEF (untreated)	0	0	0	170
RYC + CEF (X irradiated)	0	0	0	0
RYC + CEF (sonicated)	0	0	0	0

Determined from the mean number of foci on 3–4 Petri dishes
n t = not tested

Attempts to Absorb Antiviral Serum Activity by Cell Preparations

Like the the majority of mammalian Rous tumours cell free preparations from the tumours used in this investigation have given no evidence of viral activity (*Jonsson* 1966) As it could be objected that filtration or sonication of the cell preparations to obtain a cell free material might

TABLE 2

Results of Neutralization Tests Performed with Antiserum after Absorption with Material from Mammalian Rous Sarcomas or RSV Preparations

Antiserum	–	Pooled chicken serum				–	Chicken serum No 51			
Antiserum dilution		1/20	1/20	1/20	1/50		1/10	1/10	1/10	1/25
Absorbed with	–	–	rat tumour RR C190	10FTU SR RSV	–	–	–	mouse tumour RS57D	chicken tumour 1673	–
Number of foci	195	8	7	47	40	143	7	12	33	129

The figures indicate the mean numbers of 3 4 Petri dishes Neutralization tests performed with 0 1 ml SR RSV pool diluted 1 10 and 0 1 ml antiserum after absorption at 37 C for 30 min

remove or destroy small amounts of virus especially if these were in a labile form attempts were made to demonstrate viral activity by means of absorption technique Antiviral immune sera in different dilutions including the highest dilution giving clear cut virus neutralization were tested for their remaining antiviral activity after absorption for 30 min at 37 C with cell material from different mouse and rat tumours frozen and thawed repeatedly three times The results are presented in Table 2 from which it is apparent that there is no demonstrable decrease in the neutralizing capacity i e the tumour cell material cannot absorb any antiviral activity from the immune serum Negative results were also obtained with similarly treated material from mixed cultures in which the CEF were X ray damaged

The sensitivity of the procedure was tested by including SR RSV virus in small amounts or chicken tumour material treated as above as control It is apparent from Table 2 that a reduction of the virus neutralizing capacity of the serum dilution can be obtained with the chicken tumour material as well as with a virus dilution containing about 10 biologically active virus particles

Effect of Antiviral Immune Serum on Mixed Cultures

In view of the possibilities that the number of foci of transformed CEF in the mixed culture system might be dependent upon or at least influenced by a formation by the mammalian tumour cells of small amounts of virus alternatively very labile virus particles it seemed of interest to investigate the effect of antiviral immune serum of the focus formation in the system used This would be expected to prevent the spread of virus during the cultivation in liquid medium and thus also help to clarify the question whether virus is formed during the early phase (up to 4–6 days) of co cultivation either by infected CEF (after contact) or by temporarily formed heterokaryons

Table 3 illustrates the effect on mixed cultures (CEF + irradiated RR 6690 cells) of antiviral antiserum at concentrations that give clear cut virus neutralization The serum was present as long as the cultures were kept in fluid medium (up to the day after passage) but was omitted from the agar medium It is apparent from the table that the number of foci of transformed chicken cells in the cultures are of about the same magnitude irrespectively of whether antiserum is present or not Thus there is no indication of any free virus neutralizable by the immune serum participating in this early phase of the experiment

DISCUSSION

The present results confirm and extend those obtained in previous studies Thus it is not only impossible to demonstrate any viral activity in cell free material from the different mammalian tumours inves

demonstrate a transfer of the viral genome indicate that such a cell to cell contact is a very rare spontaneous event *in vitro* (once per 10^4—10 cells) but that probably one contact making tumour cell might form contacts with more than one chicken cell

SUMMARY

The mechanism of the induction of virus production in mixed cultures of Rous mouse or rat sarcoma cells and chicken embryonic fibroblasts was studied by means of irradiation of the chicken cells attempts to absorb antiviral activity with cell preparations and co cultivation in the presence of antiviral immune serum Irradiation of the chicken cells in mixed cultures prevented the production of virus No decrease of antiviral activity of the immune serum could be demonstrated by means of preparations from mammalian tumour cells in spite of the presence of specific transplantation antigens The presence of anti serum *in the mixed cultures did not significantly affect the number* of foci of transformed chicken cells formed after passage

The results indicate that RSV synthesis cannot be induced in the rodent tumour cells by mere contact with chicken cells and that the rodent tumour cells are not capable of synthesizing any viral antigen demonstrable by absorption technique It seems improbable that the foci registered after passage of the mixed cultures within 4–6 days *derive from virus released from rodent tumour cells or temporarily* surviving heterokaryons the number of foci reflects probably the number of chicken cells that have directly received the viral information via some type of cell to cell contact

REFERENCES

1 *Ahlstrom C G* Neoplasms in mammals induced by Rous chicken sarcoma material Nat Cancer Inst Monogr *17* 299–319 1964

2 *Ahlstrom C G & Lindberg L G* Growth of Rous hamster sarcoma in the chorioallantoic membrane Acta path microbiol scand 77 49 50 1969

3 *Boyse F A Old L J & Thomas C* A report on some observation with a simplified cytotoxic test Transpl Bull 29 63–67 1962

4 *Bryan W R Moloney J B & Calnan D* Stable standard preparations of the Rous sarcoma virus preserved by freezing and storing at low temperatures J Nat Cancer Inst *15* 315–329 1954

5 *Fink M A & Rauscher F J* A simple method for the preparation of potent chicken anti Rous sarcoma virus serum J Nat Cancer Inst 26 [illegible] 1961

6 *Jonsson N* Studies on the presence of the viral genome in Rous virus induced mouse tumours Acta Univ Lund Sectio II No 23 1966

7 *Jonsson N & Sjögren H O* Further studies on specific transplantation antigens in Rous sarcoma of mice J Exp med *122* 403 421 1965

8 *Lindberg L G & Jonsson N* Types of contacts *in vitro* between Rous mouse tumour cells and chicken fibroblasts as studied with the electron microscope Acta path microbiol scand 73 359–368 1968

9 *Rubin H* A virus in chick embryos which induces resistance *in vitro* to infection with Rous sarcoma virus Proc Nat Acad Sci *46* 1105 1119 1960

10 *Rubin H Cornelius A & Fanshier L* The pattern of congenital transmission of an avian leucosis virus Proc Nat Acad Sci *47* 1058 1069 1961

11 Rubin H & Temin H M A radiological study of cell virus interaction in the Rous sarcoma Virology 7 75–91 1959
12 Sheilyaghin V V & Martirosyan D M Reproduction of Rous virus in mouse embryonic tissue *in vitro* Fol Biol 12 184–188 1966
13 Šimkovič D Valentova N & Thur o V An *in vitro* system for the detection of the Rous sarcoma virus in the cells of the rat tumour XC Neoplasma 9 104 106 1962
14 Svoboda J Malignant interaction of Rous virus with mammalian cells *in vivo* and *in vitro* Nat Cancer Inst. Monogr 17 277–298 1964
15 Svoboda J Chyle P Šimkovic D & Hilgert I Demonstration of the absence of infectious Rous virus in rat tumour XC whose structurally intact cells produce Rous sarcoma when transformed to chicks Folia Biol 9 77–81 1963
16 Svoboda J Hložanek I & Machala O Quantitative assay system for the transmission of RSV genome from virogenic mammalian cells into the chicken cell with the help of Sendai virus Fol Biol 14 26–33 1968
17 Temin H M Studies on Carcinogenesis by avian sarcoma viruses V Requirement for new DNA synthesis and for cell division J Cell Physiol 69 3–64 1966
18 Temin H M & Rubin H Characteristics of an assay for Rous sarcoma virus and Rous sarcoma cells in tissue culture Virology 6 669–688 1958
19 Vigier Ph & Montagnier L Persistence and action of the Rous sarcoma virus genome in transformed rodent cells In Subviral carcinogenesis p 156–175 Ed Y Ito Kyoto 1966
20 Vigier Ph & Svoboda J Étude en culture de la production du virus de Rous par contact entre les cellules du sarcome XC du rat et les cellules d'embryon de poule Compt Rend Acad Sci (Paris) 261 4278–4281 1965

Acta path microbiol scand 77 66–72 1969

The Fourth Department of Medicine and the Connective Tissue Research Unit Second Department of Pathology University of Helsinki Helsinki Finland

ENDOCARDIAL FIBROELASTOSIS IN DOGS

By

OTTO WEGELIUS and ROBERT VON ESSEN

Received 6 iii 69

Endocardial fibroelastosis with partial aortic stenosis has previously been reported only once i e in a young Poodle (*Eliot, Jr et al* 1958) In a series comprising 5000 dogs 800 of which were examined post mortem no case of endocardial fibroelastosis was observed (*Luginbuhl & Detweiler* 1965)

Patterson (1965) described a series of 212 dogs with congenital cardiac defects The majority (131 dogs) were examined post mortem In 162 dogs a single lesion was present while the remainder exhibited complex malformations Patent ductus arteriosus and pulmonary stenosis were the most frequent findings Aortic stenosis which occurred in 29 cases came next in frequency Malformations were more common in dogs of pure breed than in mongrel dogs No sex predisposition could be demonstrated As regards the frequency of congenital cardiac defects in general dogs of Boxer breed came fifth but in respect to aortic stenosis they occupied the second place after German Shepherd Endocardial fibroelastosis did not occur

Experimental endocardial fibroelastosis has been induced in dogs by ligation of the cardiac lymphatics (*Kline et al* 1964)

This paper is a report on a litter of Boxers with congenital heart defects *In one case* definite endocardial fibroelastosis was also present

MATERIAL AND RESULTS

The dogs were of Boxer Borealis breed The pedigree of this boxer *family is shown in Fig* 1 Three litters of the family have been examined Obvious congenital cardiac defects occurred in all of them So far only male puppies have died of heart *disease* The litter of eight puppies with which this study is concerned was thoroughly analysed because one male puppy *showed endocardial* fibroelastosis at autopsy The parents of this litter were clinically healthy Three puppies were female *and five* were male Among the latter two died of cardiac failure *at the age of five months* One died at the age of two years and a half of *cerebral anoxia due to aortic stenosis*

One male puppy was killed because of underbite *His heart was not examined*

Aided by grants from the *National Research Council of Medical Sciences* and the *Sigrid Jusélius Foundation*

Pedigree of a Boxer Family with Congenital Cardiac Defects

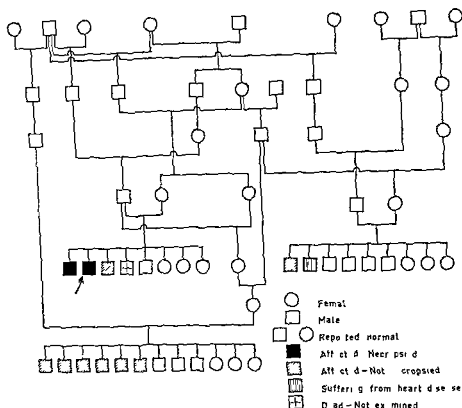


Fig 1

Case No 1

On post mortem examination of this 5 months old dog the nutritional condition was found to be satisfactory Marked stenosis of both the aorta and the pulmonary valves was observed The walls of both ventricles were hypertrophic and conspicuous necroses *were present in th left* ventricular musculature In particular th parietal papillary muscle showed extreme necrosis and calcification Fluid was present in the thoracic cavity foamy oedematous fluid was found in the trachea and the lungs *were markedly* hyperaemic and oedematous Stasis was observed in the liver and kidneys Microscopically fibrosis and degenerative changes as well as calcifications were seen in the cardiac musculature Specimens from lung kidney and liver showed stasis and hyperaemia

The possible occurrence of fibroelastosis cannot be discussed in this case It can only be established that this puppy succumbed to the sequelae of congenital stenosis of the aorta and pulmonary valves

Case No 2

This dog also died at the age of five months showing symptoms of cardiac failure Autopsy was not performed, but most probably congenital heart defect was present in this case too



Fig 2

Abundance of fine elastic fibers in the thickened semilunar valve Weigert's resorcin fuchsin stain × 650

Case No 3

This dog reached the age of two years and a half He was small for a male dog of the breed From the age of one year he showed symptoms of cerebral circulatory disturbance On physical strain he lost consciousness as a rule for some three to four minutes With increasing age these seizures became more frequent They occurred when the dog was attacked by a German Shepherd when he ran after a person on horseback when he chased a cat and when he fell from a boat into cold water Over the aorta a loud coarse systolic murmur was heard He died from a seizure when he had tried in vain to gather speed on glossy autumnal ice

At autopsy the heart was found to be enlarged The aortic orifice was constricted by a massive hard subvalvular ring of connective tissue The semilunar valves were thickened The endocardium of the dilated markedly hypertrophic left ventricle was yellowish white and shiny The remainder of the cardiac structures showed no pathological features *Stasis was not observed in any organ*

Microscopically the subvalvular ring exhibited dense collagenous tissue and hyaline cartilage The semilunar valve showed collagen deposits and a conspicuous network of fine elastic fibres (Fig 2) The endocardium showed marked thickening and an abundance of collagen and elastin The elastic fibres which in parts were coarse were mainly found in the deeper layers adjacent to the muscular tissue (Fig 3) Dense collagenous bundles invaded the musculature of the wall (Fig 4) No inflammatory cells were seen in any specimen It was impos

Performed by *I Saxen* M D professor of pathology



Fig 3
Thickened fibroelastic endocardium of the left ventricle The elastic fibers are seen as black spots and lines
Weigert s resorcin fuchsin stain X 100

sible to decide whether the lymphatics were dilated because the heart had not been pretreated with contrast stain No necroses were discernible in the myocardial specimens

This dog thus showed rather severe subvalvular aortic stenosis due to a ring of fibrotic and cartilaginous tissue in conjunction with obvious endocardial fibroelastosis in the left ventricular wall

DISCUSSION

A primary and a secondary form of fibroelastosis are distinguished (*Andersen & Kelly* 1956 *Kelly & Andersen* 1956) The primary form occurs in man as a congenital lesion without any other malformation of the heart According to *Rosahn* (1955) fibroelastosis in man is in half the cases combined with other congenital anomalies Stenosis of the aortic valve alone or stenosis of the aorta in conjunction with mitral stenosis are the most frequent findings in secondary fibroelastosis

A secondary form of fibroelastosis in the dog has been described (*Eliot Jr et al* 1958) but in this species the lesion has not been observed in its primary form without any concurrent cardiac defect By

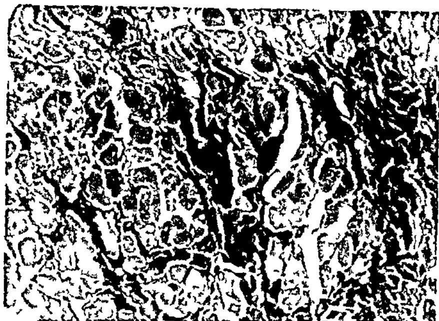


Fig 4
Collagen bundles in the myocardium of the left ventricular wall
Van Gieson stain × 650

contrast physiological fibroelastosis has been reported in animals with a large diastolic heart volume Black-Schaffer and coworkers (*Black Schaffer et al* 1965 *Grinstead & Black Schaffer* 1965) observed endocardial fibroelastosis in the sea lion hippopotamus elephant and blue whale The endocardium of these animals may show fibroelastosis changes without a presence of any malformation of the heart This phenomenon can probably be regarded as a primary form

Many aetiologic factors have been discussed in connection with fibroelastosis It seems possible that a direct mechanical cause is involved A maldirected strong jet of blood produces endocardial changes at the site where the jet hits the inner wall Histologically these changes resemble those seen in fibroelastosis (*Davies* 1960 1962) The mechanical explanation offered by *Black Schaffer* (1957) is highly acceptable and probably applies in any event to secondary fibrosis

Protracted mechanical irritation of mesenchymal structures causes increased deposition of collagen *i e* fibrosis If the structures where this occurs are subjected to continuous variations in tension elastic fibres are produced (*Bunting* 1939) Mechanical factors induce the production of elastic elements during foetal life as well (*Wegelius* 1952) Hence it seems possible that the connective tissue elements the fibroblasts in the endocardium in particular respond to increased pressure and altered variations in pressure by forming a fibroelastic tissue

In dog no 3 in the present series the endocardial changes may thus be regarded as a result of the protracted influence of mechanical factors

An increase in extracellular fluid *i e* a mesenchymal oedema develops into a mucinous organized oedema In this collagen and occasionally elastin as well are deposited The induction of lymphatic oedema in the heart produces fibroelastosis in the endocardium (*Miller et al* 1960 1963) Obstruction of the lymphatic drainage from the heart of iatrogenic or inflammatory origin causes endocardial lesions in the dog that are identical with primary fibroelastosis in man In the present series no signs of inflammation were observed

Particular interest has been attached to the possible role of mumps virus as an aetiologic factor Some investigators (*Noren et al* 1963 *St Geme et al* 1965 *Shone et al* 1966) seem to have found an association while others (*Vosbergh et al* 1965) are doubtful on this point and still others (*Gersony et al* 1966 *Grutheroth* 1966) have adduced negative results as evidence against this hypothesis In this connection it is noteworthy that mumps parotitis has been observed in dogs (*Noice et al* 1959) Coxsackie virus infection must also be taken into account as a possible causative factor (*Fruhling et al* 1962 *Mehrizi et al* 1965) The possibility of virus endomyocarditis as a cause of primary fibroelastosis cannot be eliminated However all investigators are agreed that inflammatory cells are lacking in the affected areas

Certain results have been reported which seem to indicate that fibroelastosis is a genetically determined hereditary disease (*Rosahn* 1955 *Kelly & Andersen* 1956 *Winter et al* 1960) A varying response of the connective tissue cells to mechanical irritation and/or oedema may very well be genetically determined This would also afford an explanation of the relative infrequency of endocardial fibroelastosis notwithstanding the common occurrence of congenital malformations

The fact that in the present material obvious fibroelastosis was detected in only one heart showing congenital malformation argues in favour of individual variations in the mesenchymal reaction But owing to the great difference in age at death no definite conclusions can be drawn on this point In the present case of fibroelastosis varying elevated pressure in the left ventricle certainly was a precipitating cause Mechanical irritation of the endocardium seems to be the most plausible explanation of all cases of secondary fibroelastosis With respect to the primary form the occurrence of such a factor is not so readily demonstrated Hence it seems that if pressure is normal the development of fibroelastosis is best accounted for by the presence of oedema of varying origin during a susceptible growth period in the life of the individual

In this family of boxers only male puppies have been reported dead in heart disease If the congenital defect really is sex linked many interesting questions arise However at this stage no definite con

clusions can be drawn regarding a possible sex linkage of the disease Further studies are required to ascertain that the defect does not occur in female dogs

SUMMARY

In a litter of eight puppies which were the offspring of healthy dogs of pure Boxer Borcalu breed three out of five male puppies died of congenital cardiac defect two at the age of five months and one at the age of two years and a half Autopsy was performed in two cases In a five month old puppy stenosis of both the aorta and the pulmonary valve was observed but no fibroelastosis The other puppy that died at the age of five months showed similar symptoms of heart failure but no post mortem examination was performed The dog that reached the age of two years and a half showed subaortic stenosis and definite endocardial fibroelastosis of the left ventricle Aortic stenosis and fibro elastosis in conjunction in the dog have been described only once before Mechanical factors may act as a precipitating cause of the secondary form of fibroelastosis In the present case a mechanical cause was the only one that could be demonstrated

REFERENCES

1 *Andersen D H & Kelly J* Pediatrics *18* 513–538 1956
2 *Black Schaffer B* A M A Arch of Pathol 63 281–306 1957
3 *Black Schaffer B Cranstead C F & Braunstein J V* Circulation Research *16* 383–390 1965
4 *Bunting C H* A M A Arch Path 28 306–312 1939
5 *Davies J N P* Am Heart J 59 600-627 1960 Cardiologia 4? 161–175 196?
6 *Eliot Jr T S Eliot F P Fushbaugh C C & Slager V T* J Am Vet Med Ass *133* 271–274 1958
7 *Fruhling L Korn R Laudlaureix J Surjus A & Foussereau S* Ann Anat Path (Paris) 7 2?7–303 1962
8 *Gersony W M Kat S J & Nadar A S* Pediatrics 37 430–434 1966
9 *Cranstead C E & Black Schaffer B* Lab Invest *14* 571–57? 1965
10 *Grutheroth W C* Pediatrics *38* 309 1966
11 *Kelly J & Andersen D H* Pediatrics *18* 539–555 1956
12 *Kline J K Miller A J Pick R & Kat L N* Circulation *30* 728 735 1964
13 *Luginbühl H & Detweiler D K* Ann N Y Acad Sci 127 17–540 1965
14 *Mehrizi A Hutchins G M Medearis D N & Rowe R D* Circulation Suppl II 32 p 150 1965
15 *Noice F Bolin F & Eveleth D F* Amer J Dis Child 98 350–35? 1959
16 *Noren G R Adams Jr P & Anderson R C* J Pediatr 6? 604–606 1963
17 *Patterson D F* Ann N Y Acad Sci *127* 541–563 1965
18 *Rosahn P D* Bull N Y Acad Med *31* 453–47? 1955
19 *Shone J D Armas S M Manning J A & Keith J D* Pediatrics 37 423 439 1966
20 *St Geme Jr J W Noren G R & Adams Jr P* New Engl J Med 27? 339 347 1965
21 *Vosbergh J B Diehl A M Liu C Lauer R M & Fabiyi A* Amer J Dis Child *109* 69–73 196
22 *Wegelius O* Comm Biol Soc Scient Fenn 8 (Thesis) Helsinki 1959
23 *Winter S T Moses W S Cohen N J & Naftalin J M* Amer J Dis Child *99* 529–533 1960

Acta path microbiol scand 77 73-89 1969

Ullevål Hospital Department of Pathology (Head Kristen Arnesen M D)
University of Oslo Oslo Norway

CUSHION LIKE INTIMAL LESIONS AND PLATELET AGGREGATES IN INTRAMYOCARDIAL ARTERIES OF MAN

By

JØRGEN W HÆREM

Received f ili 69

In transverse sections of intramyocardial arteries of man cushion like intimal thickenings are frequently seen They are characterized by sub endothelial deposits of a structureless material Such changes have been demonstrated previously in intramyocardial (2 8 27 49) and other smaller arteries (1 9 12 38)

Several authors (2 9 27) consider the possibility that the cushion like lesions are derived from blood borne material *Blumenthal et al* (2) described a cushion like intimal lesion as a thrombotic inflam matory lesion *Duguid & Anderson* (9) believed that the intimal thick enings were possibly derived from fibrin or that they were deposits of hyaline or amyloid substances *James* (27) described a cushion like lesion with the non committal term an older luminal occluding lesion

In rabbits organization of platelet micro emboli (19 37) small blood clot emboli (20) and fibrin emboli (22 42) have resulted in intimal cushions or intimal polyps Thus the cushion like intimal lesions in the intramyocardial arteries of man may possibly derive from earlier episodes of micro emboli

The purpose of this study was to investigate whether these cushion like intimal thickenings in human intramyocardial arteries represent organized thrombi In a consecutive autopsy series intramyocardial arteries were studied Evidence of a thrombogenic origin of the lesions was particularly searched for *e g* the occurrence of recent platelet ag gregates or more advanced thrombo emboli in the intramyocardial arteries

---

Requests for reprints should be addressed to *Jorgen W Hærem* Ullevål Hospital Department of Pathology Oslo 1 Norway

This work has been supported by grants from *the Norwegian Council on Cardio vascular Diseases* and from *J L Tidemanns Tobaksfabrik–Johan H Andresens me I ønske fond*

Leif Jorgensen M D has given help and advice Technical assistance has been produced by *Liv Lovlik*

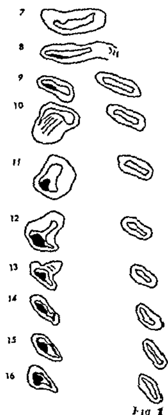
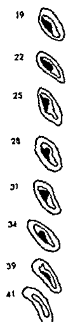


Fig 1



Fig 2

Figs 1–2

Fig 1 Reconstruction by serial sectioning of a myocardial artery bifurcation The figures refer to the section number A cushion like lesion (black) originates at the bifurcation and extends distally in one of the arteries Note the localization of the lesion to the lateral aspect of the fork

Fig 2 Based upon serial sections a schematic reconstruction of a branching myocardial artery is shown A cushion like lesion (black) is seen at both sides of the orifice of the branch (1) Longitudinal section side view (2) Longitudinal section viewed from above (3) Cross section at the level of the branch Intimal lips are shown (See Fig 4)

In a succeeding publication the relation of the cushion like lesions to age sex coronary atherosclerosis and certain diseases will be discussed

MATERIAL AND METHODS

The consecutive autopsy series consisted of 129 males and 86 females from 1 to 96 years of age One hundred and twenty two men and 84 women were 40 years of age and older the median age of men and women was 65 and 73 years respectively

The autopsies were performed 8 to 34 hours after death Myocardial samples measuring 2 by 2 by 0 3 cm were taken for histological examination from (a) the left side of the interventricular septum and (b) from the lateral wall of the left ventricle close to and parallel to the endocardium This part of the myocardium contains many small arteries (c) Samples of the upper third of the interventricular septum were cut perpendicular to the atrioventricular valve rings and included all the wall layers This plane of section was expected to produce many cross sections of the intraseptal arteries (d) Samples were also taken without preference

from both ventricles of randomly selected hearts (e) In some randomly selected cases sample adjacent to the blocks (b) and (c) were prepared for frozen sections
In 14 randomly selected cases sections from kidneys liver and pancreas were studied
Paraffin sections were prepared from blocks fixed in 10 per cent unbuffered formaldehyde solution The following stains were used Haematoxylin and eosin (HE) Mallory's phosphotungstic acid haematoxylin (PTAH) Lendrum's martius scarlet blue (MSB) method for fibrin (30) Lendrum's martius scarlet direct blue method for the detection of old fibrin (31) Gomori's aldehyde fuchsin Verhoeff's stain Bernhold's Congo red and Gomori's reticulin stain Gelatin embedded frozen sections were stained with Sudan III for lipids Some tissue blocks containing many intimal cushions were sectioned serially
One section from the blocks (a) or (b) and one from the block (c) were screened systematically with a magnification of 100 One of the sections (d) in randomly selected cases was screened in order to compare the prevalence of intimal cushions in different parts of the ventricles

Definitions Bifurcation Site of division of one artery into two arteries both of which have about the same calibre and both leaving the main stem at about the same angle
Branch Relatively small artery leaving a considerably larger main stem

RESULTS

Cushion Like Intimal Lesions

These lesions were observed in one or more myocardial arteries in the majority of cases older than 40 years and in none of the nine cases younger than this They were rarely observed in arteries larger than 150 microns or in arterioles smaller than 30 microns in diameter The frequency of affected arteries was about the same in various sections from both ventricles Such lesions were also present in epicardial arteries and in some sections from the kidneys liver and pancreas However other types of arterial lesions were more prominent in the abdominal organs

Serial sections revealed that the intimal lesions usually were elongated ridges almost spindle shaped running along the longitudinal axis of the arteries for a distance of 100 to 500 microns or more depending on the size of the vessel These lesions were frequently observed at and distal to bifurcations often at the lateral aspect of the circumference in the fork (Fig 1) The lesions were also frequent at and distal to orifices of arterial branches Here they were usually found in the main stem only Some intimal lesions close to orifices of arterial branches appeared in cross sections as intimal lips (Fig 2 and 4) However the lesions could not always be definitely related to any bifurcation or branch A possible localization of intimal lesions to arterial bends was difficult to reconstruct In cross sections of the arteries the cushion like lesions often encroached considerably on the lumen of the vessel (Fig 3) Rarely however did the thickened intima appear to occlude the artery completely In general the intimal lesions were larger and protruded more in hearts with a high frequency of lesions



Figs 3–4

Fig 3 Myocardial artery with a large cushion like intimal lesion Haematoxylin *and eosin stain* × *1150*

Fig 4 A cross section of a myocardial artery at the level of a branch (see Fig 2) Paired intimal thickenings appear as lips close to the orifice of the branch Note the orderly arrangement of smooth muscle cells Small amounts of weakly stained subendothelial structureless material is seen in the lower lip (arrows) Haematoxylin and eosin stain × 500

In sections stained with HE the intimal lesions appeared as sub endothelial deposits of a bright red structureless or sometimes coarse fibrillar material Endothelial cells were often absent in the region of the lesions (Fig 6 and 10) and often in other parts of the arteries as well The endothelial defects were equally extensive in cases where autopsy was performed shortly after death and in cases where autopsy was performed later Infrequently detached endothelial cells were ob served in the arterial lumina (Fig 9)

The intimal lesions appeared to have a relation to the elastic tissue of the arterial wall In cross sections the non occluding protruding

lesions tapered gradually at one or both sides and merged with the internal elastic membrane (Fig 6 and 14) Verhoeff s stain and Gomori s aldehyde fuchsin stained some of the structureless material in the intimal lesions black and dark purple respectively Also the coarse fibrillar material seen with HE stained as elastin In the region of the lesions disruption and splitting of the internal elastic membrane had usually taken place (Figs 7 and 8) Most evident in cushions of the smallest arteries small cells were often encountered in close association with the disrupted and split elastic membranes (Figs 7 and 8) Elastic material structureless or with a beaded appearance was often located at the periphery of these cells (Fig 7) Elastic fibres were sometimes abundant suggesting new formation Thus in the region of intimal lesions both disruption and building up of elastic fibres seem to take place

Small lipid droplets were observed in the deeper parts of some lesions They were seen under and between the lamellae of the disrupted and split internal elastic membrane The lipid was mainly extracellular (Fig 5) but lipid droplets were also present in small cells To a lesser extent lipid occurred in the media and then only in regions of intimal lesions

Very rarely the structureless material stained in patches as fibrin with the MSB and PTAH stains

Congo red for amyloid did not stain the structureless material of the cushion like intimal lesions

Both the coarse fibrillar and the structureless material gave fairly strong reaction with PAS probably indicative of mucopolysaccharides in the lesions Alcian blue demonstrating acid mucopolysaccharides gave an inconstant and weak stain

Fibres staining as collagen were found in small amounts in the larger lesions (Fig 10)

In many intimal cushions small cells were observed to be irregularly scattered In the larger lesions they were almost invariably present In many lesions they were elongated with a dense ovoid nucleus (Fig 5) The cytoplasm was rather scanty and with PTAH it stained as the smooth muscle cells of the media These cells are probably smooth muscle cells It was noted that cells of this type were orderly arranged (Fig 4) in some intimal lips at orifices of arterial branches Some of these "lips contained the structureless material whereas others did not

Other cells in the intimal cushion like lesions were rounded uncharacteristic (Fig 5) and their cytoplasm did not react with PTAH They could be undifferentiated intimal cells or mononuclear cells of haematogenous origin (Fig 9) The cells containing lipid and the cells related to disrupted elastic fibres in the smaller cushions were mostly of this type

In HE sections the tunica media underneath the intimal cushion







7

like lesions usually showed no change although sometimes there was a thinning of the media with possible disappearance of muscle fibres

Although marked perivascular fibrosis was seen in many hearts there was no constant relationship between perivascular fibrosis and intimal cushions

Intimal lesions in myocardial arteries with a certain resemblance to the described cushion like lesions were occasionally found in areas adjacent to and within areas of old and healing myocardial infarcts The lesions were found in arteries measuring 100 up to 300 microns or more in diameter In cross sections they often encroached considerably on the lumen of the vessels In serial sections they appeared to be elongated as the cushion like lesions described above Their relation to arterial bifurcations and branches could not be evaluated because the number of such lesions was small In some instances serial sectioning of the lesions revealed that they represented thrombi in various stages of organization

A study of the relation of these lesions to the internal elastic membrane of the arterial wall failed to reveal tapering of the lesions and merging with the internal elastic membrane The internal elastic membrane had often disappeared underneath the lesions Elastic fibres were seen in these lesions but the amount was smaller than in the intimal cushion like lesions described above Structureless material reacting with the elastic stains was not seen

Some parts of these lesions containing young connective tissue showed greater cellularity than the intimal cushion like lesions (Fig 11) They probably contained both smooth muscle cells and fibrocytes The cellularity of the older lesions of this type was of the same order as in the cushion like lesions or somewhat larger (Fig 12)

Considerable amounts of PAS positive material was demonstrated in the lesions containing young connective tissue whereas the older lesions of this type stained only weakly

Underneath these older lesions medial fibrosis was often seen (Fig 12) This was rare in the intimal cushion like lesions

---

Figs 5-7

Fig 5 A cushion like lesion Small smooth muscle cells (S) and possibly undifferentiated cells (M) in the intima Lipid droplets (F) are seen in the deeper intimal layer and in the media mainly extracellularly The media is thinned and muscle fibres have disappeared L = arterial lumen A = tunica adventitia Sudan III stain × 1650

Fig 6 Large cushion like lesion tapers to one side Lendrum s MSB stain × 700

Fig 7 Two cushion like lesions in a small myocardial artery The internal elastic membrane is split at the lower lesion In the upper lesion there may be new formation of elastin around the undifferentiated cell (M) Note the beaded appearance of the elastin at one side of the cell (arrow) and the structureless mass of elastin at the opposite side of the cell Gomori s aldehyde fuchsin stain × 1440

Platelet Aggregates and Platelet Emboli

Only platelet aggregates larger than 10–15 microns were observed with the smaller magnification (× 100) The bulk of aggregates had diameters larger than 50 microns In all cases where platelet aggregates were observed at least one aggregate was larger than 50 microns in diameter

Platelet aggregates smaller than 10–15 microns in diameter were occasionally found when the larger magnification (× 400) was applied Since the screening for platelet aggregates was carried out at the magnification of 100 nothing can be concluded about the prevalence of the smaller platelet aggregates

Platelet aggregates in myocardial arteries were found in eight cases out of the total number of 215 cases (3 7 per cent Table 1)

TABLE 1

The Eight Cases with Platelet Aggregates in Myocardial Arteries (m a.)

Case	Sex	Age	Clinical data	Pathological findings in the coronary arteries and the myocardium
1	M	34	Alcoholism Pneumonia Recent myoc inf	Fresh occluding coronary thrombus and myocardial infarct Several platelet aggregates in m a
2	M	79	Prostatic carc Uraemia Br pneumonia	Small myocardial infarcts of varying age A Platelet aggregate in one m a
3	M	79	Recent myoc inf	Severe coronary atherosclerosis Fresh occluding coronary thrombus Platelet aggregates in several m a
4	M	52	Old myoc inf Chronic heart failure	Old myocardial scar Small platelet aggregates in two m a
5	F	76	Traffic accid Shock Dead two weeks later	A platelet aggregate in one m a
6	F	75	Chronic heart failure	A platelet aggregate in one m a
7	F	61	Pulm emboli	A platelet aggregate in one m a
8	F	77	Cerebral thrombosis	Severe coronary atherosclerosis Platelet aggregates in a few m a and in small veins Possibly early myocardial necrosis

The size of the arteries where aggregates were found varied from 75 to 350 microns in diameter

The platelet aggregates in the two cases with recent upstream thrombi (cases 1 and 3 Table 1) were more numerous than in the other cases and some occluded the arteries completely (Fig 13) Some aggregates in these two cases contained small amounts of fibrin



Figs 13 14

Fig 13 Occluding platelet aggregates in two myocardial arteries Lendrum's MSB stain × 190

Fig 14 A small platelet aggregate (arrows) in contact with a cushion like lesion Platelets appear to adhere to endothelial cell and to an area denuded of endothelium (P) Two red blood cells are close to the platelet aggregate Hematoxylin and eosin stain × 840

The platelet aggregates in the other six hearts were few and non occluding they appeared to float in the vessel lumen

Platelet aggregates larger than 10–15 microns in diameter were only rarely seen to be in contact with the cushion like lesions Some occluding larger aggregates were seen to be situated at branchings of vessels

By randomly applied higher microscopical magnification in these eight cases recent mural platelet thrombi were discovered to be in

contact with intimal cushions in a very few instances These thrombi measured only a few microns (Fig 14)

Organizing thrombi in myocardial arteries were not seen in these eight cases

In myocardial veins, platelet aggregates were seen in four cases In three cases they were observed to be in relation to infarcted or possibly infarcted myocardium Only one of the cases is included among the eight cases above (case 8) In the fourth case there was no heart disease and the patient died from acute pyelonephritis

DISCUSSION

Morphology of the Intimal Cushion Like Lesions

As possible sources of elements that build up the intimal cushions both (a) the blood, and (b) the arterial wall must be considered

(a) There was some evidence of plasma substances in the intima Some of the lipid demonstrated in the lesions may have been derived from the plasma similar to the lipid in large arteries (4 11 21 33) Further in a couple of instances fibrin staining material was observed in the lesions This material could be either fibrin or fibrinogen (35)

(b) The PAS positive reaction of the intimal deposits would include the possible presence of a number of substances among others basement membrane material Elastin was also demonstrated in the lesions Modified smooth muscle cells are known to produce these substances (3 15 17 41 50) In larger arteries these cells also accumulate and liberate lipid into the arterial wall (15 17) Even the smallest intimal cushions contained cells which could be small smooth muscle cells or undifferentiated mesenchymal cells These cells therefore probably play a role in the production of the cushion like lesions Their origin could be migrating multifunctional medial cells (15 50)

Thus both the blood and the arterial wall may contribute to the elements which build up the cushions but it is likely that the bulk of the material originates from the vessel wall

Platelet Aggregates, Platelet Emboli, and Organizing Thrombi

Platelet aggregates larger than 10–15 microns in diameter may have been overlooked in some cases and many aggregates smaller than 10–15 microns may not have been recognized because of the small magnification applied for screening Only a small part of the total myocardium of each heart was examined Therefore it is likely that the real frequency of platelet aggregates in an unselected autopsy series is higher than that observed

In two cases (cases 1 and 3) it is likely that the platelet aggregates represent dislodged and embolized thrombus material In six hearts no acute lesions in the main epicardial coronary arteries were found

lesions in myocardial arteries However there was no constant relationship between perivascular fibrosis and the cushion like lesions Further the muscular layer of the arteries was usually well preserved The opposite would have been expected if inflammation had taken place

(d) The most likely explanation of the lesions is that they are the result of constant or repeated trauma to the vessel wall The frequent localization of the cushion like lesions as bifurcations at orifices of arterial branches and the occurrence of subendothelial deposits upon intimal lips suggest that the blood flow may play a role in the production of the lesions

In tubes laminar flow is disturbed at branchings and bends (14 18) and eddy formation may occur (14) Disturbed blood flow is associated with endothelial injury (16) At bifurcations at orifices of branches and at bends of large arteries endothelial injury has been demonstrated in swine and rabbits (39) At sites of disturbed blood flow in guinea pigs increased endothelial regeneration seems to take place (10) This indicates that a disturbed blood flow in large arteries may represent a constant trauma to the vessel wall at predilection sites

It cannot be excluded that the endothelial defects in the myocardial arteries partly were due to postmortal detachment of endothelial cells In fact some defects evidently were due to autolysis or artifacts But on the other hand because endothelial cells only infrequently were found to be shed in the arterial lumina it is likely that many endothelial defects had occurred during life This suggests that endothelial injury occurs also in the smaller myocardial arteries

The endothelial injury may lead to increased plasma diffusion and subendothelial accumulation of plasma derivatives as in large arteries (39) Supporting evidence of intimal injury may be found in the observation of an occasional white blood cell in some of the intimal lesions

The exact mechanism of injury to the vessel wall is not clear The formed elements of the blood including the platelets may play a role (28 39) The presence of floating platelet aggregates in myocardial arteries of some hearts and the finding that platelets occasionally are in contact with the intimal lesions may further indicate that the platelets are involved in the mechanism of injury Such a mechanism could include a reversible and repeated interaction between platelets and the vessel wall

SUMMARY

In 215 consecutive autopsies the myocardium was examined in order to explore the pathogenesis of cushion like intimal thickenings in the intramyocardial arteries

Predilection sites of the lesions were at bifurcations and branching sites of small arteries measuring 30 to 150 microns in diameter The

lesions were elongated spindle-shaped intimal protrusions rich in elastic material. Besides they contained collagen fibres, mesenchymal and sometimes mononuclear blood cells, lipid and very rarely fibrin staining material.

The most likely explanation of the lesions is that they are the result of intimal injury due to haemodynamic trauma to the vessel wall.

There was no evidence that the lesions represented organized thrombo emboli.

Platelet aggregates in intramyocardial arteries were observed in eight hearts (3 7 per cent). Platelets are supposed to be involved in the mechanism of intimal injury.

REFERENCES

1 *Bell E T & Clawson B J* Primary (Essential) hypertension A study of 420 cases Arch Path 5 939–1002 1928
2 *Blumenthal H T Alex M & Goldenberg S* A study of lesions of the intramural coronary artery branches in diabetes mellitus Arch Path 70 13–28 1960
3 *Buck R C* Histogenesis and morphology of arterial tissue In Sandler M & Bourne G H eds Atherosclerosis and its Origin Acad Press New York and London 1963 Pp 1–38
4 *Connor W E* Dietary sterols Their relationship to atherosclerosis J Amer diet Ass 52 202–208 1968
5 *Conti G* Sur la morphologie des anastomoses artério veineuses et des dispositifs régulateurs du courant sanguin Ann Anath path 3 5–32 1958
6 *Cox G E & Wexler B C* Platelet and fibrin thrombi in isoproterenol induced myocardial necrosis Fed Proc 27 413 1968
7 *Davis R B* Electron microscopic changes in blood platelets induced by bacterial lipopolysaccharide Exp molec Pathol 5 559–574 1966
8 *Donomae I Matsumoto Y Kokubu T Koide R Kobayashi R Ikegami H Ueda E Fujisawa T & Fujimoto S* Pathological studies of coronary atherosclerosis Especially of sclerosis of intramuscular coronary arteries Jap Heart J 3 423–441 1962
9 *Duguid J B & Anderson G S* The pathogenesis of hyaline arteriolosclerosis J Path Bact 64 519–522 1952
10 *Eades G H & Sevitt S* Microthrombosis in injured and burned patients J Path Bact 93 275–293 1967
11 *Field H Jr Swell L Schools P E Jr & Treadwell R* Dynamic aspects of cholesterol metabolism in different areas of the aorta and other tissues in man and their relationship to atherosclerosis Circulation 22 547–558 1960
12 *F hb g A M* Anatomic findings in essential hypertension Arch intern Med 3 650–668 1925
13 *Fourman J & Moffat D B* The effect of intra arterial cushions on plasma skimming in small arteries J Physiol (London) 158 374–380 1961
14 *Fox J A & Hugh A F* Localization of atheroma A theory based on boundary layer separation Brit Heart J 28 388–399 1966
15 *French J E* Atherosclerosis in relation to the structure and function of the arterial intima with special reference to the endothelium In Richter G W & Epstein M A eds International Review of Experimental Pathology Acad Press New York and London 1966 Pp 253–353
16 *Fry D L* Acute vascular endothelial changes associated with increased blood velocity gradients Circulat Res 22 165–197 1968
17 *Geer J C McGill H C Jr & Strong J P* The fine structure of human atherosclerotic lesions Amer J Path 38 263–288 1961

18 *Gutstein W H Schneck D J & Marks J O* In vitro studies of local blood flow disturbances in a region of separation J Atheroscler Res 8 381-388 1968

19 *Hand R A & Chandler A B* Atherosclerotic metamorphosis of autologous pulmonary thromboemboli in the rabbit Amer J Path 40 469-486 1962

20 *Harrison C V* Experimental pulmonary arteriosclerosis J Path Bact 60 289-293 1948

21 *Haust M D More R H Bencosme S A & Balis J V* Electron microscopic studies in human atherosclerosis Extracellular elements in aortic dots and streaks Exp molec Pathol 6 300-313 1967

22 *Heard B E* An experimental study of thickening of the pulmonary arteries of rabbits produced by the organization of fibrin J Path Bact 64 13-19 1952

23 *Hirsch H & Caethgens P* Die Entstehung von Thrombocytenaggregaten durch Extremitatenischemie beim Hund Z ges exp Med 139 227-237 1965

24 *Hudson R E B* Cardiovascular Pathology 1 ed vol 1 Edward Arnold Ltd London 1965 Pp 531-571

25 *Hughes A & Tonks R S* The role of micro emboli in the production of car ditis in hypersensitivity experiments J Path Bact 77 207-217 1959

26 *Hughes A & Tonks R R* Intravascular platelet clumping in rabbits J Path Bact 84 379-390 1962

27 *James T N* Pathology of small coronary arteries Amer J Cardiol 20 679-691 1967

28 *Jorgensen L Rowsell H C Hovig T Glynn M F & Mustard J F* Adenosine diphosphate induced platelet aggregation and myocardial infarction in swine Lab Invest 17 616-644 1967

29 *Jorgensen L Rowsell H C Dodds W J & Mustard J F* Myocardial infarction in an old dog J Atheroscler Res 8 97-110 1968

30 *Lendrum A C Fraser D S Slidders W & Henderson R* Studies on the character and staining of fibrin J clin Path 15 401-413 1962

31 *Lendrum A C Fraser D S & Slidders W* Further observations on the age changes in extravascular fibrin Ned T Geneesk 108 2373 1964

32 *Lui B R Fulton G P & Akers R P* White thromboembolism in hamster cheek pouch after trauma infection and neoplasia Circulation 3 339-351 1951

33 *McGill H C Jr & Geer J C* The human lesion Fine structure In Jones R J ed Evolution of the Atherosclerotic Plaque University of Chicago Press Chicago and London 1963 Pp 65-76

34 *McKay D G Margaretten W & Csavossy I* An electron microscope study of the effects of bacterial endotoxin on the blood vascular system Lab Invest 15 1815-1829 1966

35 *Moe N & Abildgaard U* Histological staining properties of in vitro formed fibrin clots and precipitated fibrinogen Acta path microbiol scand 6 61-73 1969

36 *Moffat D B* An intra arterial regulating mechanism in the uterine artery of the rat Anat Rec 134 107-121 1959

37 *Moore S & Mersereau W A* Platelet embolism and renal ischemia J Path Bact 90 579-588 1965

38 *Moritz A R & Oldt M R* Arteriolar sclerosis in hypertensive and nonhypertensive individuals Amer J Path 13 679-728 1937

39 *Packham M A Rowsell H C Jorgensen L & Mustard J F* Localized protein accumulation in the wall of the aorta Exp molec Pathol 714 232 1967

40 *Payling Wright P* Endothelial mitosis around aortic branches in normal guinea pigs Nature 220 78-79 1968

41 *Pease D C & Paule W J* Electron microscopy of elastic arteries the thoracic aorta of the rat J Ultrastruct Res 3 469 483 1960

42 *Rabin F R Thomas W A Lee K T Konikov N & Scott R F* The microembolism and experimental systemic arteriosclerosis Arch Path 64 75-81 1957

43 *Reale E* & *Luciano L* Elektronmikroskopische Beobachtungen an Stellen der Astabgabe der Arterien Angiologica (Basel) *3* 276–239 1966
44 *Saphir O* *Ohringer L* & *Wong R* Changes in the intramural coronary branches in coronary arteriosclerosis Arch Path *62* 159–170 1956
45 *Schulz H* & *Landgraber E* Elektronenmikroskopische Untersuchungen über die Adsorption und Phagocytose von Influenza Viren durch Thrombozyten Klin Wschr *44* 998–1006 1966
46 *Silver M D* & *Stehbens W E* The behaviour of platelets in vivo Quart J exp Physiol *50* 241–247 1965
47 *Stetson C A Jr* Studies on the mechanism of the Shwartzman phenomenon Certain factors involved in the production of the local hemorrhagic necrosis J exp Med *93* 489–504 1951
48 *Wagenvoort C A* Die Bedeutung der Arterienwulste fur den Blutkreislauf Acta anat (Basel) *21* 70–99 1954
49 *Wegelin C* Ueber Arteriosklerose im Myocard Schweiz med Wschr *74* 57–60 1944
50 *Wissler R W* The arterial medial cell smooth muscle or multifunctional mesenchyme? Circulation *36* 1–4 1967

The Chromosome Laboratory Department of Pathology Kommunehospitalet Copenhagen (Heads Professor Hemming Poulsen M D and Dr Per Christoffersen M D) and the Fuglebakken Children s Hospital Copenhagen (Head Dr Henning Andersen M D)

A VARIANT CHROMOSOME IN THE 13–15 GROUP TRANSMITTED THROUGH THREE GENERATIONS

By

JAKOB VISFELDT and HENNING ANDERSEN

Received 22 III 69

The large acrocentric chromosomes in the 13–15 group will frequently show translocations Assessed on the basis of the number of reports available, they will be involved considerably more frequently than other chromosomes It should be realized however that it is easier to reveal lesser structural aberrations in the 13–15 group than *e g* in chromosomes in the 6–12 group

The types of translocations concerned which have all been described in several reports are classified naturally into three groups translocations of whole chromosomes (*e g* the D/D translocation) translocation of chromosome material to the long arm and translocation of material to the short arm As regards the last group the conditions are complicated by the fact that the size of the satellites may vary considerably If the short arm is unusually elongated it will of course be difficult to decide whether it is a normal variation or whether it is a question of translocation of sparse material

The object of the present paper is to report on the study of a family in whom a variant chromosome was found in the 13 15 group with a marked increase in the normal length of the short arm This variant chromosome was found in 7 of 13 members of the family examined through three generations The nature and origin of the chromosome aberration described are discussed and its clinical implications are evaluated

---

We are indebted to Miss *Grethe Jensen* Cand Pharm for expert technical assistance

The work was assisted by grants from the *Statens Almindelige Videnskabsfond*

We should like to thank Dr *Hjalmar Larsen* M D Orthopedic Hospital Copenhagen for allowing us to study the propositus.

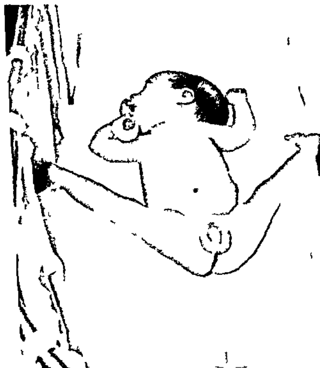


Fig 1

The child at 3 months Flexion contractures in hip joints and hands and equino varus position of the left foot

MATERIAL AND METHODS

The propositus in this study is a boy born in March 1968 of a 25 year old primi gravida. Both the mother and father are healthy of normal intelligence and with out any physical or mental abnormalities

The child was born at the expected term after a normal pregnancy in particular had no infections nor any other known noxious exogenous factors been present Because of breech presentation with prolapse of the umbilical cord caesarean section was carried out

At birth it was observed (Fig 1) that both legs were lying along the body with hip joints flexed The hips could be extended passively to 30 Both legs could be moved freel —there were no contractures of any other joints of the lower extre mities Moderate equino varus position of the left foot was observed

Both hands were clenched The fingers were remarkably slim There was flexion contracture of the first second and third fingers whilst the fourth and fifth fingers could be moved fr ely There were bilateral mongolian creases and missing distal bending crease of the third finger Remaining examinations showed normal findings

The child was re examined at the age of 12 months His mental development cor responded to his chronological age Only slight flexion contracture remained in the hips which could otherwise be moved freely but he was not able to stand on his legs The hands were still clenched but the fingers could be stretched although with some difficulty (Figs 2 and 3) The thumbs were the most difficult to stretch they were typically adducted into the palms

When the boy was 1? weeks old chromosome analysis on peripheral blood was carried out employing the ordinary micromethod with a culture period of 48 hours A total of 20 cells was analysed all of them showing a variant chromosome in the

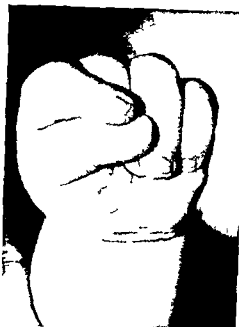




Fig 2 *Fig 3*

Figs 2 and 3

The child at 1 *year Still flexion contracture of the hand On passive stretching* the mongolian creases and the missing distal bending crease of the third finger are seen

13–15 group The chromosome analysis of the child was repeated and chromosome analysis on peripheral blood from the parents was carried out employing the ordinary macro method with a culture period of 48 hours It appeared that the father was a carrier of the same chromosome aberration as the child The mother s karyotype was *normal Subsequently chromosome analyses on peripheral blood* from the father s family were carried out (Fig 4) We could not obtain blood from II 1 and her chilrren III 1 2 3 as this branch of the family had emigrated.

From all samples at least 20 cells were analysed

RESULTS

The pedigree appears in Fig 4

It appears that identical chromosome aberrations were found in 7 of the 13 members from three generations of the family who were examined All the persons except the propositus were phenotypically normal and presented no malformations they were found to be of normal intelligence without any mental abnormalities It was ascer*tained that no spontaneous abortions had occurred in the family*

The variant chromosome which was found in all the cells analysed from the 7 carriers is a member of the 13–15 group with normal length of the long arm On the basis of measurements it appears to be a chromosome No 14 or No 15 The short arm is always seen with the normal secondary constriction and corresponding to the position of the satellites extra material was observed in an *amount almost equal* to the *long arm of* a G chromosome The chromosome material is of

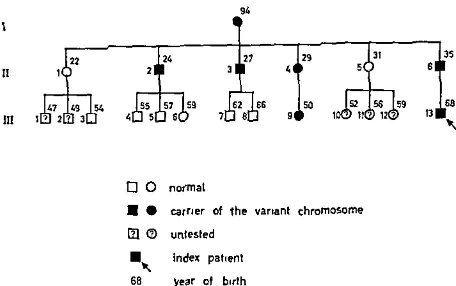


Fig 4
Pedigree of the family

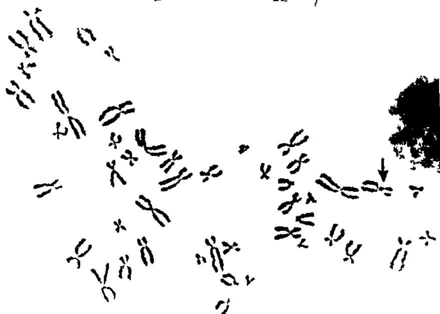


Fig 5
Mitosis from propositus (Fig 4 III 10) Chromosom s of the 13–15 group indi ated



Fig 6

Mito is from father of propositus (Fig 4 II 6) Chromosomes of the 13–15 group indicated

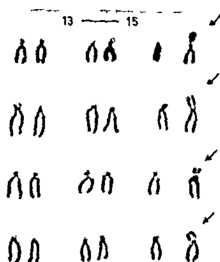


Fig 7

Chromosomes of the 13–15 group from four different carriers (Fig 4 II 3 4 f) The variant chromosome indicated

normal density and contour and in some cells the chromatids are lying parallel

Idiograms were produced of a great number of mitoses from the persons concerned in particular from the propositus his father and his paternal grandmother but it was impossible to demonstrate absence of chromosome material on other chromosomes

Figs 5 and 6 show photographs of mitoses from the child and from the father

In Fig 7 the chromosomes in the 13–15 group are shown derived from cells from 4 carriers of the variant chromosome

DISCUSSION

The identification of the variant chromosome did not present any difficulties As mentioned above, the chromosome aberration was found in all analysed cells in the carriers and in all well suited cells the secondary constriction on the chromosome is very distinct (Figs 5 6 and 7) No other aberrations in the karyotypes were found neither in the carriers nor in the remaining persons examined The ratio of carriers to non carriers of the aberration is not different from 1 1

On the basis of morphological studies the following possibilities as to the nature of the chromosome aberration have been considered

a) D/G translocation in Down s syndrome
b) normal variation of the short arm of a D chromosome
c) partial trisomy after a translocation

Re a) The clinical and the cytogenetic examination of the propositus and the family makes it very unlikely that this is a case of Down s syndrome with D/G translocation However *Sergovich et al* (6) have reported on an atypical case of Down s syndrome in which the short arm of one of the chromosomes in the 13–15 group was abnormally long A study of the parents and siblings of this child revealed normal karyotypes The illustrations accompanying the paper by *Sergovich et al* indicate that the short arm of the variant chromosome contains considerably less material than is the case in our patient Furthermore the arms are not morphologically identical from one karyotype to another to such an extent as in our patient

Re b) In the literature some reports have been published on cases in which the short arm of a D chromosome has been found to be abnormally long without giving rise to phenotypical manifestations *Court Brown et al* (1) describe in a father and his son who were healthy and without obvious abnormalities unusually long short arm on one long acrocentric autosome without satellites In many cells the chromatids on the short arm presented a somewhat fuzzy appearance and tended to lie together in close approximation as do those of the long arm of the Y



Fig 6
Mito is from father of propositus (Fig 4 II 6) Chromosomes of the 13–15 group indicated

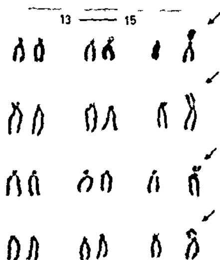


Fig 7
Chromosomes of the 13–15 group from four different carriers (Fig 4 II 2 3 4 6)
The variant chromosome indicated

of material to the short arm of a D chromosome corresponding to the satellite region itself —By mating between a translocation heterozygote and a normal partner there are four possible zygotes normal trans location heterozygote partial trisomy and chromosome deletion It is stated that no chromosome aberrations were demonstrated in the mem bers of the family other than the elongated short arm on a D chromo some in the 7 carriers When the material is so limited as in the present study it is possible that a C chromosome can be involved in the trans location in various ways without the ability to reveal this by normal chromosome analyses

When the chromosome aberration has not produced any phenotypi cal manifestations in the family of the propositus this might be due to the fact that either there are carriers only of a reciprocal translocation which does not give rise to abnormalities or if there are carriers of partial trisomy or chromosome deletion that the actual chromosome material is inactive in the persons concerned It might be believed that the structural changes in the chromosomes involved act in a com plex integrated genetic pattern Depending on this a chromosome aberration in most cases might have no phenotypical manifestations but in certain cases it might give rise to abnormalities This theory could explain why only the propositus presented abnormalities

However it should be emphasized that the relationship between the chromosome aberration found and the phenotypical abnormalities in the propositus must be interpreted as a theoretical possibility only They are fairly unspecific abnormalities which might occur in con nection with various syndromes or in otherwise normal individuals The present study does not offer adequate grounds for claiming a re lationship between the chromosome aberration and the abnormalities found in the propositus

On the basis of the clinical and the cytogenetic study of the family and the above discussion it can be concluded that the increased length of the short arm of the D chromosome *can* be a normal variant How ever because of the amount density and contour of the chromosome material it is presumably translocated material from another chromo some but it is impossible to show which chromosome is involved in this case A relationship between the chromosome aberration and the abnormalities found in the propositus is not rendered probable but on theoretical considerations it cannot be excluded

SUMMARY

Because of flexion contractures in the hips equino varus position of the left foot flexion contractures and mongolian creases of the palms chromosome analysis on peripheral blood was carried out in a boy 12 weeks old In all the cells analysed a chromosome in the 13–15 group was found the short arm of which was considerably longer than nor

mal From a family study the same variant chromosome was found in the child s paternal family in a further 6 phenotypically normal persons through three generations The nature of the chromosome aberration is discussed and it is stated that it might be a normal variation However it is considered more likely that the aberration is the result of a translocation of material from another chromosome possibly from the 6–12 group to the chromosome in the 13–15 group

A correlation between the chromosome aberration and the abnormalities found in the propositus is not rendered probable but on the basis of theoretical considerations it cannot be excluded

REFERENCES

1 *Court Brown W M Jacobs P A Buckton K E Tough I M Kuenssberg E V & Knox J D E* Chromosome studies on adults Cambridge University Press London New York 1966 Pp 12–39

2 *Grouchy J de Thieffry S Aicardi J Chetrie J J & Zucker C* Trisomie partielle C par translocation t(Cq— Dp+) et remaniement d un C(p—q+) Arch franc Pediat *24* 859–868 1967

3 *Lord P M & Cooke P* A familial variant chromosome in the 13–15 group J med Genet *4* 114–116 1967

4 *Makino S Muramoto J I & Tabata S* A survey of familial transmission of an anomalous autosome in group 13–15 Chromosoma (Berl) *18* 371 379 1966

5 *Migeon B R & Young W J* Reciprocal (D F) translocation euploid transmission in three generations Bull Johns Hopk Hosp *115* 379–383 1964

6 *Sergovich F R Valentine G H Carr D H & Soltan H C* Mongolism (Down s syndrome) with atypical clinical and cytogeneti features J Pediat 6 197–207 1964

Acta path. microbiol. scand. 77: 99–109, 1969

The Pediatric Research Laboratory, Variety Club Heart Hospital, University of Minnesota, Minneapolis, and Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois

CHANGES IN THE CONCENTRATION OF LYMPHOCYTES IN THE INTESTINAL EPITHELIUM OF HIBERNATING GROUND SQUIRRELS (*CITELLUS TRIDECEMLINEATUS*)

By

K. E. FICHTELIUS[1] and B. N. JAROSLOW

Received 14.iii.69

The significance of the presence of lymphocytes between the epithelial cells of the intestine has generally been regarded as an indicator of the cellular response to invasion by enteric organisms. In recent years the origin and disposition of these cells have been studied (2). They seem to be immigrants from the blood, constituting a selection of young lymphocytes compared to the ordinary mixture of mostly old blood lymphocytes. It has also been shown that some of them reenter the lamina propria (8). *Fichtelius* (1, 2) has suggested that the intestinal epithelium may function as a first level lymphoid organ making the lymphocytes immunologically competent. Recent discoveries in the phylogeny of lympho-epithelial relationships have created a need for additional knowledge of lymphocytes within the intestinal epithelium (3, 4).

Attention has recently been given to the status of immune mechanisms of hibernating animals (5). It has been shown that although the processes associated with antigen disappearance are very slow during hibernation, the events of the latent period, namely induction, maturation and proliferation, can take place during this period. The final antibody production takes place after arousal.

The new interest in lymphocytes of the gut epithelium and in the immune mechanisms of hibernating animals justifies an investigation of the occurrence of lymphocytes within the epithelium of duodenum and ileum of normal and hibernating ground squirrels (*Citellus tridecemlineatus*). The preliminary findings indicate that these lymphocytes, here called theliolymphocytes, accumulate in above normal amounts during hibernation.

This work was supported by the U.S. Atomic Energy Commission.

[1] Visiting professor of the American Heart Association, present adress: Department of Histology, University of Uppsala, Sweden.

MATERIAL AND METHODS

The 47 ground squirrels used in this study were collected in northern Illinois during the summer of 1967 and were used in January and February 1968. They were kept in plastic cages and fed Rockland Guinea Pig Diet supplemented with pecans. They were housed in a room on a 12 hour light cycle. To induce hibernation the ground squirrels were placed in clear bakelite cylinders (13 inches wide and 16 inches high) that were half filled with shavings and placed in a dark room maintained at 5° C and 50 per cent relative humidity (5). The body temperature of the animals in the cold room was continuously recorded from the output of a small transmitter implanted in the peritoneal cavity. It decreased to 8.5° C when the animals were in hibernation (See *Kayser* (7) for a discussion of hibernation). Sham transmitters were implanted in the euthermic controls.

These experiments took place in the same animals that were given an intraperitoneal injection of 1.6×10^9 sheep red cells during an investigation of the immune response in hibernating ground squirrels. These treatments did not induce changes in the theliolymphocyte count that could be associated with the immune response that developed, i.e. there was no increase in theliolymphocytes with time.

When the animals were sacrificed samples of the duodenum and the ileum were collected, fixed in formalin, sectioned and stained with haematoxylin eosin. At least one thousand epithelial nuclei were counted in sections from every specimen and the number of theliolymphocytes per 1000 epithelial nuclei was recorded. Only the epithelium from perpendicularly sectioned villi was examined and in every *instance the extreme tip of the villus was avoided. All counting was done by a* specially trained technician who was unaware of the design of the experiment.

RESULTS

Twenty four ground squirrels were placed in the 5° C room to hibernate and 6 groups of 4 were sacrificed at 2, 5, 6, 8, 9 and 11 days after entry into the cold room. An equal number of euthermic controls were sacrificed at the same time with the exception that 3 were sacrificed in the 11 day controls. The number of theliolymphocytes per 1000 epithelial cells was counted in the duodenum at 6 time points and in the ileum at 5 time points (See Fig. 1). The mean ± the standard error of the number of theliolymphocytes per 1000 epithelial cells per section was determined from 24 sections of duodenum and 20 sections of *ileum from* 24 hibernating animals. The mean was 149 ± 11 and 134 ± 11 respectively. From 23 euthermic controls we made similar measurements in 23 sections of duodenum and 19 sections of ileum. The means were 115 ± 9 and 119 ± 7 respectively. These results indicate that there is a larger than normal concentration of theliolymphocytes in the duodenum of the hibernators than in that of the controls ($P < 0.02$). The theliolymphocyte count between the ileums is also higher in the hibernators *but the difference gives* a $P > 0.5$ by Student's *t* test.

DISCUSSION

These results indicate that the concentration of theliolymphocytes is above normal in the intestine of hibernating ground squirrels. More lymphocytes may be coming to the epithelium per unit time than usual at a constant transit time for lymphocytes *within the epithelium*. The number of incoming lymphocytes may however be constant or even

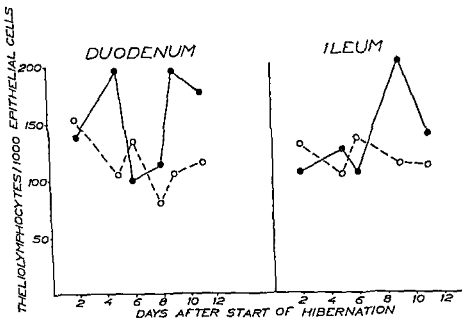


Fig 1

The theliolymphocytes/1000 epithelial cells in the villi of ground squirrel intestine examined at different times after ground squirrels were placed in hibernation solid line Euthermic controls are shown by the broken lines Each point represents the mean of 4 animals in the group All hibernators and their controls were injected with sheep red cells one to three days before hibernation Note that there is no increase in the theliolymphocyte count in the controls in response to the sheep red cells The late increase of these cells in the hibernators is therefore related to hibernation

lower than normal at a prolonged transit time This latter alternative seems more reasonable than the first since the general effect of hibernation is to slow down most processes The functional capacity of the epithelium as a first level lymphoid organ may be directly dependent upon the number of lymphocytes which pass the epithelium and return to lamina propria per unit time not upon the number of lymphocytes within the epithelium at a certain time

It is unlikely that the increased number of theliolymphocytes in hibernating animals is due to stimulation by a low grade infection because as has been demonstrated (6) bacterial invasiveness is severely inhibited by hibernation

SUMMARY

A new interest in lymphocytes of the gut epithelium and in the immune mechanisms of hibernating animals justifies an investigation of the occurrence of lymphocytes within the gut epithelium of normal and hibernating animals Twenty four ground squirrels (*Citellus tridecem lineatus*) were placed in hibernation for periods up to 11 days The

concentr ition of lymphocytes was examined in the epithelium denum and ileum of hibern itors and euthermic controls sac different times during this interval A significant increase cytes within the epithelium was observed in the hiberr

REFERENCES

1 *Fichtelius K E* The Mammalian Equivalent to B Cell Res 46 231-234 1967
2 *Fichtelius K E* The Gut Epithelium—a Fir t l Res 49 87-104 1968
3 *Fichtelius K F Finstad J & Good R A* brates To be published in Laborat
4 *Fichtelius K F Finstad J & Good R* phocytes within the Gut epitl
5 *Jaroslow B N & Smith D I* Eff *normal and Irradiated Gr* m
6 *Kalabukhov N I* Characteristics of h Factors in their Sensitivity to I I Immunobiol 29 1453-1460 19 8
7 *Kayser C* The Physiology of Natural Hi NY 1961
8 *Meader R D & Landers D F* Electron and Light lationships between Lymphocytes and Intestin *121* 763-774 1967

Acta path microbiol scand 77 103-116 1969

Department of Histology Institute of Human Anatomy Uppsala Sweden

THE LYMPHO EPITHELIAL ORGANS OF HOMO SAPIENS REVISITED

By

K E Fichtelius C Sundström B Kullgren
and J Linna

Received 14 iii 69

The lymphoid organs have been classified in different ways A recent classification speaks about central or first level lymphoid organs and peripheral or second level lymphoid organs The first level lymphoid organs defined so far are the thymus and the bursa Fabricii The removal of these first level organs early in ontogenesis leads to a deficient development of the second level lymphoid organs that is the main rest of the lymphoid organs Thymus and bursa Fabricii are both lympho epithelial organs in the sense that they represent an intimate relationship between epithelial or epithelial derived cells and lymphocytes

In birds thymus seems to be responsible for the development of that part of the lymphoid system which exerts cellular immunity (5) Bursa Fabricii seems to be responsible for the development of that part of the lymphoid system which exerts immunoglobulin production (5)

All animals phylogenetically above the lamprey have a thymus and a thymus dependent function *i e* cellular immunity (14) All animals phylogenetically above the lamprey display the bursa dependent function *i e* immunoglobulin production but only birds have a bursa Fabricii (14) The search for a bursa equivalent in bursaless vertebrates has gone on for some years in Dr Robert Good s laboratory There is growing evidence that the Peyer s patch type of tissue in rabbits an other lympho epithelial organ has a bursa function (4 5)

Partly based on the statements mentioned above about the phylogeny of adaptive immunity partly based on old and new observations on lymphocytes within gut epithelium *Fichtelius* advanced a theory according to which the epithelium of the gut in bursaless vertebrates is a first level lymphoid organ the epithelium having the same influence on lymphocytes and lymphoid tissue as has the bursa Fabricii in birds (8) The mammals may according to this theory be in the process of developing a special bursa equivalent the epithelium covering the gut

---

Requests for reprints should be adressed to K F Fichtelius Department of histology *Institute of human anatomy* Uppsala Sweden

The work has been supported by the *Swedish Medical Research Council*

associated lymphoid tissue But even in mammals the epithelium of the entire gut with its lymphoid cells may function as a bursa Fabricii (8)

The new concept of first level and *second lymphoid organs* and the fact that the first level lymphoid organs defined so far are lympho epithelial gives a new meaning to the old classification of the lympho epithelial organs as a particular entity among the lymphoid organs It also justifies a new close look at all types of lympho epithelial relationship in a phylogenetic and ontogenetic perspective This paper is a revisit to the lympho epithelial organs of homo sapiens It is based on *studies of the literature* and on new observations in newborn children As a review it is very incomplete —thymus is not even mentioned It stresses some new aspects on the lympho epithelial organs Some formations earlier described otherwise and probably *misinterpreted* will be presented as lympho epithelial organs A few new lympho epithelial organs will also be described

MATERIAL AND METHODS

Nine newborn infants and two foetuses were examined Some of the significant data on the material are summarized in Table 1 From most of the cases the following organs were taken for histology larynx anal region salivary glands (parotid submandibular sublingual) the middle part of pancreas oesophagus main bronchi toe nail region preputial fornix vaginal wall fornix and portio lower conjunctival fold external ear canal lower ear lobe scrotum and areola mammae In addition the duodenal part of pancreas was examined from two newborn children otherwise not included in the material

The sections were 5 μ every 20th section was collected stained with haematoxylin eosin and examined

RESULTS

The Tonsils

The aperture by which the oral cavity communicates with the next portion of the digestive tract the pharynx contains accumulations of lymphatic tissue within the mucous membrane Besides small infiltrations with lymphocytes which may occur anywhere in this part of the mucous membrane well outlined organs are formed by the lymphatic tissue The surface epithelium invaginates into them There are the lingual tonsils the palatine tonsils and the pharyngeal tonsils Their *histology is too well known to be described here In* this connection it must be emphasized however that these organs are well developed at birth They can certainly not be looked upon as the consequence of inflammation as far as the individual is concerned

The tonsils may be mixed lymphoid organs in the sense that they are both first level and second level *Surjan* (31) has cultured tissue from the palatine tissue of homo sapiens in vitro and proven it to be antibody forming This does *not necessarily mean* that the tonsils are second level organs Thymic tissue produces tetanus antitoxin when

TABLE 1

Presentation of the Material

Number	Sex	Time of delivery before (—) or after (+) expected delivery	Time of death before (—) or after (+) delivery	Cause of death
1	♂	—60 days	— 1 day	Mother appendectomized *Immaturity* respiratory insufficiency
	♂	0	+ 1 day	Congenital heart malformation (ventricle septal defect) diaphragma hiatus hernia pneumothorax respiratory *distress* syndrome
3	♀	—80 days	— 1 day	Myomatosis uteri Immaturity
4	♀	Premature Weight at delivery *1600 g*	+ 6 days	Multiple malformations Immaturity Circulatory insufficiency
5	♂	—10 days	+ 2 days	Intestinal obstruction *Respiratory* distress syndrome Circulatory insufficiency
6	♂	—60 days	+ 1 day	Respiratory distress syndrome Bleeding from vena terminalis
7	♂	0	+ 19 days	Mongolism. Operated *rectal atresia* Respiratory and circulatory insufficiency
8	♂	—35 days	+ 4 days	Mongolism Congenital heart malformation Duodenal atresia, Pneumonia
9	♂	—20 days	—few hours	Abruptio placentae *Immaturity*
10	♀	Length 27 cm	0	Abortion in month V–VI
11	♀	Length 19 cm	0	Abortion in month IV–V





Fig 1

Fig 1a A small number of lymphocytes accumulated under the laryngeal epithelium of a foetus 100 × (case 10)

Fig 1b An accumulation of lymphocytes under the laryngeal epithelium [illegible] born 100 × (case 8)

transplanted into the anterior chamber of the eyes of irradiated mice (30) and thymus is at least looked upon as only first level

Other tonsils are less well known The tonsils of Eustachian tubes take the whole circumference of the pharyngeal end of the tube and confluences with the pharyngeal tonsil This tissue is as a rule well developed in newborn (19) The tonsil of the larynx is situated in the laryngeal ventricle and on the false vocal cords It is said to appear in four months old children (17)

In our material collection of lymphocytes in close relation to the epithelium of larynx was observed in cases 2 5 7 8 9 and 10 The very small accumulation of lymphocytes in the foetus (case 10) is shown in Fig 1 as well as the larger accumulation in one of the newborns (case 8)

The so called rectal tonsil may be better described below among the Peyer s patch type tissue

The Peyer s Patch Type Tissue

There is a large amount of literature about the lymphatic tissue of the gut (25) The ontogeny and phylogeny of the Peyer s patch tissue has been discussed in recent years by Good and associates (4 14) Since these organs thus already have been revisited from our current point of view the comments here will be very brief

As already stated there is growing evidence that this tissue may have a bursal function at least in rabbits (6 14) It does however probably also function as a second level lymphoid organ What part of these formations is doing what remains to be demonstrated

It is perhaps less well known that the amount of lymphoid tissue in rectum is increasing towards anus (25) and has even been described as the rectal tonsil (28) The lymphoid tissue in this region sometimes proliferates in the same way as the tonsils of pharynx and these proliferations have often been classified as benign lymphomas (16 18 20)

The lymphoid tissue around anus seems to be well developed already in newborn children In our material accumulations of lymphocytes in relation to the epithelium was discovered in cases 1 2 3 5 9 and 10 The lymphocyte accumulations in the foetus (case 10) as well as one of those in one newborn (case 9) is shown in Fig 2 It is evident from the figures that such accumulations of lymphocytes occur under stratified as well as under cylindrical epithelium

Diffuse Lympho epithelial Relationship of the Intestine

There are lymphocytes in nearly all epithelia in the body In the epithelium of the small intestine in man there are 5–10 per cent They occur very early in ontogeny already in 9 week old embryos (26) The kinetics of these cells have been studied in rats with the aid of H_3 thymidine labelling (9) The results of these experiments can best be





Fig 2

Fig 2a Peyer's patch type tissue from the anal region of a foetus 100 × (case 10)
Fig 2b An accumulation of lymphocytes immediately under the squamous epithelium of the anal region of a newborn 100 × (case 9)



Figs 3–4

Fig 3 A primitive intraparotid lymph n le of a foetus containing glandular tissue 100 × (case 10)

Fig 4 A large accumulation of lymphocytes around serous acini and ductuli of pancreas of a newborn 100 × (case 3)

explained by an immigration of a selection of young lymphocytes from the blood It has long been a belief that most of the lymphocytes of the gut epithelium are lost into the lumen Ultrastructural studies of the gut have shown however that a least some of these lymphocytes are heading back into lamina propria (22)

Lymphoid Tissue in the Salivary Glands, Including Pancreas

The *occurrence of lymph nodes in the interlobular* tissue of the parotid gland of homo sapiens has been known for a long time (19) and was pointed out to us by *Talal (32)* These lymph nodes very often contain parotid gland tissue (24) There are regularly 8–14 of these lymph nodes within the parotid glands of newborn children (24) The glandular tissue included in the lymph nodes has been classified as lympho epithelial lesions of different kinds either inflammatory or neoplastic (13) in spite of the fact that they are present in apparently normal foetuses

In our material *intraparotid lymph nodes containing parotid gland* tissue have been found in cases 2 3 4 5 8 9 10 and 11 Fig 3 shows *a developing intraparotid lymph node from the older of the foetuses* (case 10)

The submandibular and sublingual glands do not seem to contain many of these lymph nodes with glandular tissue but such structures have been described in the submandibular gland (13) In our material such a lymph node was found only in the submandibular gland of case 2 However large accumulations of lymphocytes were observed around the duct from the sublingual gland in cases 4 7 and 10

Large accumulations of lymphocytes were seen in the middle part *of the exocrine pancreas in cases 3 4 5 8 9* and 10 (Fig 4) These accumulations did not have the characteristics of encapsulated lymph *nodes In the two extra cases from which the duodenal end of pancreas* was examined there were several such accumulations of lymphocytes Lymphocyte accumulations in the normal pancreas *of the kind de*scribed here have not been reported hitherto as far as we know

The Lymphoid Tissue of Oesophagus

Accumulations of lymphocytes can be seen around the excretory ducts of the mucous glands of oesophagus in man (7 12) They often *have a very characteristic relationship* to the ducts and the glands as is very clear from Fig 5

In our material accumulations *of lymphocytes were observed* in cases 6 7 8 and 11 but the relationship to the glandular epithelium was not as clear cut *as stated by Flesh (12) Other mammals have* much more of lymphoid tissue in oesophagus these formations have been called folliculi tonsillaris oesophagi in pigs (27)

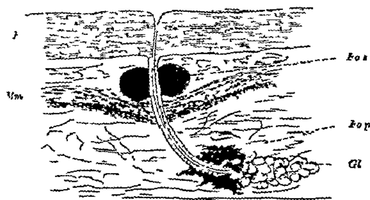


Fig 5

A schematic drawing by *Flesh* 1888 illustrating the lymphocyte collections related to the mucous glands of oesophagus The lymphoid tissue is indicated by F o s and Fo p E = epithelium Mm = muscularis mucosae Gl = mucous gland

The Lymphoid Tissue of the Bronchi

At each bronchial bifurcation or trifurcation the rat lung usually shows a cellular formation of variable size which has been called the peribronchial lymphoid tissue focus (3) These foci usually reach the bronchial epithelium and the lymphocytes are infiltrating the epithelium (3)

Such collections of lymphocytes are very common also in the human lung (2 23) It seems however as if the lymphoid tissue of the human lung does not make contact with the epithelium as often as in the rat lung (2) Newborns or foetuses whether of human subjects or of rats were not examined

In our material only a small piece of the main bronch was examined Lymphoid tissue was found in cases 1 2 3 4 and 8 but a close spatial relationship between the lymphoid tissue and the epithelium was not observed in any case The lungs of newborn children and foetuses certainly have to be reexamined from this point of view bearing in mind that the accumulations of lymphoid tissue are usually located at the bifurcations or trifurcations of the bronchi

Brandelet (3) concludes that the peribronchial foci of lymphoid tissue are capable of pouring out lymphocytes into the bronchial lumen He thinks that this pouring out of cells into the bronchial lumen is a physiological phenomenon The histological picture however also fits the theory advocated in this paper—that the majority of the lymphocytes near the surface may not be lost into the lumen but may reenter the circulation

Lympho Epithelial Relationships of the Skin

There are 1 4 per cent lymphocytes in the germ layer of the human epidermis (1) The function and fate of these lymphocytes are entirely



Fig 6

Lymphocyte collections around the deep glandular tissue of the external ear canal of a foetus 100 × (case 10)

unknown Experimental studies of the guinea pig indicate that a selection of young lymphocytes from the blood are accumulating in the epidermis (15 21) The epidermis as a first level lymphoid organ will be discussed elsewhere (11)

According to a theory put forward by *Fichtelius* (10 and discussion) the formation of lympho epithelial organs is induced in phylogeny by *the presence of antigen Such organs could thus be expected to be* found in locations where the antigen exposure is special or large For this reason we looked under the toe nails in the genital region at the conjunctival fold and in the external ear canal Another guess about the location of lympho epithelial organs in the skin is the predilection sites of lymphadenosis benigna cutis *i e* the lower ear lobe scrotum and areola mammae The thought behind this guess is that lymphadenosis benigna cutis is a kind of hypertrophy of lymphoid tissue comparable to the hypertrophy of the tonsils and the rectal tonsil

Under the toe nails and in the preputial fornix the lympho epithelial relationship was completely diffuse in our material There were remarkably many lymphocytes in the connective tissue underlying the epithelium of fornix vaginae and at the tip of the connective tissue papills in the rest of vagina Tiny collections of lymphocytes were observed in the connective tissue of the conjunctival fold in many cases (2 3 4 5 7 8 and 9) In one case (9) a collection of lymphocytes was observed around an accessory lacrimal gland Small collections of



Fig 7
A lymphocyte collection in connection with a sebaceous gland of the scrotal skin of a newborn 100 × (case 8)

lymphocytes were observed around the glandular tissue in the external ear canal in cases 3 4 6 8 9 and 10 Some of the collections found in the older of the foetuses (case 10) is shown as an example in Fig 6

Small collections of lymphocytes were found around hair follicles and sebaceous glands of the lower ear lobe in cases 2 and 5 There were more such collections to be seen around the glandular tissue of the scrotal skin (cases 2 6 7 and 8) Lymphocytes around a sebaceous gland are shown in Fig 7 Similar collections of lymphocytes were observed around the primitive mammary gland tissue in cases 2 6 and 10

DISCUSSION

It is logical and biological to look upon the very early phylogeny of immunity in the following way

The first place where external and internal antigens are met with by the primitive organism is the outer and inner surfaces of the body In the case of external antigens this is obvious Internal antigens (= not self) most likely originate in rapidly proliferating tissues The gut epithelium and the epidermis are the most rapidly proliferating tissues of such animals This leads to the assumption that the first antigen reactive cells were epithelial cells that the first primitive antibodies were formed by epithelial cells We know that the secretory piece or transport piece which probably is formed by epithelial cells comes before

7 S IgA in the ontogeny of man (29). Primitive antibodies produced by epithelial cells may very well precede antibodies produced by lymphoid cells in phylogeny. The lymphoid cells may have come to help at a higher level of organization when there was a selection pressure for a more effective gut function and the epithelial cells had to become free to specialize in other directions. This can be looked upon as a parallel to the development of red blood corpuscles (from the same stem cells?) coming to help the growing organism in oxygen transport.

Initially the lymphoid cells might have done their job within the epithelium itself. Later in the development they had to do it somewhere else after having received some kind of instruction from the epithelial cells. In the beginning this hypothetical instructor function of epithelial cells may have been exerted by all surface cells. When this function became concentrated to certain areas of the gut epithelium there could certainly be different instructions going on in different locations depending on the antigen which originated the instruction at the particular site. In birds there are at least two such locations, the thymus and the bursa Fabricii, offering at least two types of hypothetical instruction. In mammals there are many types of lympho-epithelial locations known. In one of them, the thymus, instruction of lymphocytes has been anticipated. There may be other types of instruction going on in all lympho-epithelial relationships.

This theory explains why the lymphocytes made contact with the epithelium in the first place and why the epithelium is leading during the development of the lymphoid system.

A number of lymphocyte collections in close relationship to epithelium have been described in this article. Most of these seem to be present in the human organism already before birth. Case 10, a foetus of 27 cm, has been shown to have lymphocyte collections with a close spatial relationship to epithelium in many locations (larynx, anal region, parotid and sublingual glands, pancreas, vaginal wall, external ear canal and areola mammae). It is less probable that all these accumulations of lymphocytes in case 10 are a consequence of inflammation. It is also very unlikely that all the other lympho-epithelial relationships met with in newborns discussed here are a consequence of inflammation. On the other hand it is reasonable to assume that we have dealt with small lympho-epithelial organs of different shape and size.

The function of these organs remains to be demonstrated. The theory just related to above represents an attempt to put them all under one hat as partial bursal equivalents.

SUMMARY

The new concept of first level and second level lymphoid organs and the fact that the first level lymphoid organs defined so far are lympho-

epithelial justifies a new close look at all types of lympho epithelial relationship in a phylogenetic and ontogenetic perspective This paper is a revisit to the lympho epithelial organs of homo sapiens It is based on studies of the literature and on new observations in newborn children and foetuses

A number of lymphocyte collections in close relationship to epithelium are described most of which seem to be present in the human organism already before birth It is less probable that all these accumulations of lymphocytes are all consequences of inflammation On the other hand it is reasonable to assume that we are dealing with small lympho epithelial organs of different shape and size The function of these organs remains to be demonstrated An attempt is made here to put them all under one hat as partial equivalents to bursa Fabricii of birds

REFERENCES

1 *Andreasen E* On the occurrence of the lymphocytes in the normal epidermis Acta Dermatovener 29 17 1952

2 *Arnold J* Über das Vorkommen lymphatischer Gewebes in der Lunge Virchow Arch. Path Anat 80 315 1880

3 *Brundelet P J* Experimental study of the dust clearance mechanism of the lung I Histological study in rats of the intra pulmonary bronchial route of elimination Acta path microbiol scand 175 1 1965

4 *Cooper M D Gabrielsen A E & Good R A* The development differentiation and function of the immunoglobulin production system In Smith R T Miescher P A & Good R A Ontogeny of Immunity University of Florida Press Gainesville Florida 1967 p 122

5 *Cooper M D Peterson R D A & Good R A* Delineation of the thymic and bursal lymphoid systems in the chicken Nature London 205 143 1965

6 *Cooper M D Perey D Y Gabrielsen A E Sutherland D E R McKneally M F & Good R A* Production of an antibody deficiency syndrom in rabbits by early removal of their organized intestinal lymphoid tissues Int Arch Allerg and Appl Immunology 33 65 1968

7 *Dohlowalski Z* Lymphknotchen (folliculi lymphatici) in der Schleimhaut der Speiseröhre des Magens des Kehlkopfs der Luftrohre und der Scheide Betr Path Anat 16 43 1894

8 *Fichtelius K E* The mammalian equivalent to bursa Fabricii of birds Exp Cell Res 46 231 1967

9 *Fichtelius K E* The gut epithelium—a first level lymphoid organ? Exp Cell Res 49 87 1968

10 *Fichtelius K E* The theliolymphoid organs—a new look at the lympho epithelial structures Exp Hematology (Oak Ridge National Laboratory) 17 III, 1968

11 *Fichtelius K E Groth O & Liden S* Epidermis—a first level lymphoid organ To be published

12 *Flesch M* Über Beziehungen zwischen Lymphfollikeln und secernierenden Drusen im Oesophagus Anat Anz 3 283 1888

13 *Godwin J T* Benign lymphoepithelial lesion of parotid gland Report of eleven cases Cancer 5 1089 1952

14 *Good R A Finstad J Pollara B & Gabrielsen A F* Morphologic studies on the evolution of the lymphoid tissues among the lower vertebrates In Smith R T Miescher P A & Good R A Phylogeny of Immunity University of Florida Press Gainesville Florida 1966 p 149

15 *Groth O* The selection of lymphoid cells in allergic contact dermatitis in guinea pigs Acta Dermatovener 44 91 1964

16 *Gruenwald P* Abnormal accumulations of lymph follicles in the digestive tract Amer J Med Sci 203 823 1942

17 *Heiss R* Der Kehlkopf In Mollendorff W v (Ed) Handbuch der Mikroskopischen Anatomie des Menschen Vol 3 Julius Springer Berlin 1936 p 798
18 *Heller E L & Lewis H H* Benign lymphoma of the rectum Amer J Path 26 463 1950
19 *Kolmer W* Die Tuba Eustachii In Mollendorff W v (Ed) Handbuch der Mikroskopischen Anatomie des Menschen Vol 1 Julius Springer Berlin 1927 p 272
20 *Li I Y* Benign lymphoma of the rectum Surgery 23 814 1948
21 *Lidén S* Epithelial cell reutilization of nuclear material and selective accumulation of lymphoid cells in allergic contact dermatitis Acta Dermato vener 47 137 1967
22 *Meader D R & Landers D F* Electron and light microscopic observations on relationships between lymphocytes and intestinal epithelium Amer J Anat *121* 763 1967
23 *Miller W S* The distribution of lymphoid tissue in the lung Anat Rec 5 99 1911
24 *Neisse R* Über den Einschluss von Parotisläppchen in Lymphknoten Anat Hefte Abt 1 H 32 B 10 H 2 1898
25 *Patzelt V* Die Follikel Peyerschen Platten und tonsillenartigen Organe des Darmes In Mollendorff W v (Ed) Handbuch der Mikroskopischen Anatomie des Menschen Vol 3 Julius Springer Berlin 1936 p 314
26 *Patzelt V* Die Histogenese des Dünndarms In Mollendorff W v (Ed) Handbuch der Mikroskopischen Anatomie des Menschen Vol 3 Julius Springer Berlin 1936 p 28
27 *Schumacher S* Die Speiserohre In Mollendorff W v (Ed) Handbuch der Mikroskopischen Anatomie des Menschen Vol 1 Julius Springer Berlin 1927 p 301
28 *Strindl H* Bartels Tonsille des Mastdarmes und ihre Stellung in der Pathologie des lymphatischen Apparates des Mastdarmes Zentralblatt f Chirurgie 62 2594 1935
29 *South M A Cooper M D Wollheim F A Hong R A & Good R A* The IgA system I Studies of the transport and immunochemistry of IgA in the saliva J Exp Med *123* 615 1966
30 *Stoner R D & Hale W M* Antibody production by thymus and Peyer s patches intraocular transplants Immunology 75 203 1955
31 *Surjan L* Personal communication
32 *Talal N* Sjogren s syndrome Bull Rheum Dis *16* 404 1966
33 *Zimmermann K W* Der Lymphapparat der Speicheldrüsen In Mollendorff W v (Ed) Handbuch der Mikroskopischen Anatomie des Menschen Vol 1 Julius Springer Berlin 1927 p 161

Acta path microbiol scand 77 117 133 1969

Rigshospitalet Medical Department P University Hospital of Copenhagen 2100 Copenhagen Ø Denmark Institute for Experimental Immunology University of Copenhagen 2100 Copenhagen Ø Denmark Blood Bank and Blood Grouping Laboratory Municipal Hospital 8000 Arhus C Denmark

STUDIES ON THE QUANTITATION OF THE LYMPHOCYTE RESPONSE *IN VITRO*

A Family Study

By

SOREN FREIESLEBEN SORENSEN
and FLEMMING KISSMEYER NIELSEN

Received 4 iii 69

In man the major histocompatibility locus which determines the most important transplantation antigens is called HL-A It seems to contain two separate mutational sites sub loci or may be two closely linked ordinary loci each containing a considerable number of mutually exclusive genes and corresponding antigens The two genetic units are called LA and 4 respectively The existence of other linked mutational sites besides these cannot be excluded

By means of serological typing of peripheral lymphocytes and thrombocytes it is possible to define a considerable number of antigens determined by the HL A locus Even though all antigens are not known at present recent investigations suggest that within the near future it will be possible to determine the majority of antigens defined by this locus (10)

HL A typing permits determination of the number of antigens in the donor which are not found in the recipient but apart from this the typing procedures do not in themselves allow determination of the antigenic strength in a given combination

In the mixed leucocyte culture technique (MLC) as originally described by *Bain Vas & Lowenstein* (3 4) leucocytes from two individuals are mixed in tissue culture and if the two individuals are

We wish to thank Dr *Fritz Bach* for helpful discussions and his suggestion of using rayon wool for separating the lymphocytes

In addition we wish to thank Dr *L Staub Nielsen* The Blood Bank and Blood Grouping Department University Hospital of Copenhagen for undertaking the HL-A typing in the P family and *Ove Ree* for computing the statistical data

Esther Wilschi is thanked for excellent technical assistance

This study was supported by *Thorsens Foundation King Christian X s Memorial Foundation* and the *Danish State Research Foundation*

genetically different mutual stimulation gives rise to blastogenesis and cell division. In one way cultures as described by *Bach & Voynow* ([illegible]) leucocytes from one of the individuals are pretreated with *mitomycin C* rendering these cells incapable of participating in the response which in this method is quantitated one week after the preparation of the cultures by measuring the cellular uptake of ^{3}H Thymidine.

A high correlation between the linked HL-A antigens and the results of the MLC test has been demonstrated (2). Most remarkable is the constant demonstration of non stimulation between HL-A identical siblings. This implies that the HL-A antigens alone determine the MLC reaction or that at any rate other antigen systems of importance must be determined by genes rather closely linked with the HL-A locus. HL-A identical cultures were clearly shown to differ from cultures obtained from HL-A non identical siblings even when these showed only low stimulation. Furthermore *Bach et al* (6) and *Albertini & Bach* (1) have presented evidence that different degrees of HL-A incompatibility may be recognized using the MLC technique. On the other hand it is of course *not possible to define individual HL-A antigens* with this matching procedure.

HL-A typing in combination with matching procedures are therefore at present necessary to evaluate the degree of incompatibility in a given combination. However attempts made in an effort to increase the quantitative precision of the MLC response have met with several difficulties due to the technical demands imposed by the test. Previous investigations indicate that a more reliable quantitation of the *in vitro* lymphocyte response could be obtained by measuring the thymidine incorporation early in the culture period instead of during the ensuing peak response (14).

The main purpose of the present work has been to apply this method in the case of family members who have been genotyped in advance with respect to the HL-A locus.

Assuming that the HL-A system contains two fairly well defined loci, *i.e.* LA and 4, each of these bearing a number of multiple allelic genes, each individual must have four HL-A genes, two LA genes and two 4 genes which determine the corresponding HL-A antigens. Due to the extensive polymorphism both loci will usually be heterozygous and each individual therefore possesses *four different genes and antigens*. By serological HL-A typing of parents and children the segregation of the different genes may be followed and the parental HL-A chromosomes of the children may be determined. If the two HL-A chromosomes of the father are designated ab and the mother's cd four different chromosome combinations (HL-A genotypes) are possible, *i.e.* ac, ad, bc and bd. For a given genotype (*e.g.* ac) there is thus the possibility of finding siblings who differ by two chromosomes (bd), by one chromosome (ad, bc) or having identical chromosomes. The parents will due to the great polymorphism usually have different

HL-A chromosomes whereas both of the parents will always share one chromosome with any of their children

If it is assumed that the response in MLC reflects the degree of the HL-A incompatibility then donors within a family differing by two chromosomes would be expected to give a stronger stimulation than donors differing from the responder by only one chromosome However in practice this prediction may not always manifest itself Thus the varying strength of individual antigens and the presence of the same genes on more than one of the parental chromosomes might mask the additive effect

MATERIAL AND METHODS

HL-A typing was performed partly using highly selected lymphocytotoxic antisera in microtechnique (11) and partly using complement fixing thrombocyte antibodies as described previously (13) The HL-A antigens which could be characterized are listed in Table 2 The majority of these antigens have been described previously (10)

The principle in the chromosome analysis performed is described by *Ceppellini et al* (7)

Mixed lymphocyte culture was performed as previously described (14) 50–150 ml of blood was defibrinated and mixed with equal volumes of the following solution 7 parts of TC 199 (Glaxo) and 3 parts of Dextran 6 per cent in 0 9 per cent sodium chloride (Intradex Glaxo) After sedimentation for 30 60 minutes the supernatant containing leucocytes was removed The sediment was centrifuged (1000 G 15 minutes) for preparation of cell free medium

In supernatant of responding cells the concentration of lymphocytes was adjusted to 1 × 10^6 per ml either after centrifugation (200 G 10 minutes) or by dilution with cell free medium

Supernatants containing stimulating cells were transferred to 50 ml syringes containing approximately 4 grammes of rayon wool (Rank Xerox Organization) After 20 minutes incubation at 37 C the eluate was expressed The non adherent cells were concentrated (200 G 10 minutes) to a volume of 6–8 ml and the supernatant used for preparation of cell free medium The cells were treated with Mitomycin C (Sigma) 25 μg per ml for 20 minutes at 37 C and washed twice (200 G 10 minutes) in medium pool from all the stimulators included in the experiment Finally the cells were resuspended in the same medium pool with a lymphocyte concentration of 1 × 10^6 per ml In four experiments a higher concentration of stimulating lymphocytes was used as described below The granulocyte contamination of the stimulating lymphocytes was 0–10 per cent

Mixed cultures were prepared from equal volumes of responding and stimulating cells Unstimulated controls were prepared from unmixed leucocyte suspension from the responders A control for the mitomycin treatment was prepared from equal volumes of stimulating cells from two HL-A non identical persons All culture volumes were 2 ml Brown screw capped medicine bottles delivered aseptic from Plåt Manufaktur Limmared Sweden were used as culture vials The gas phase of the vials was 5 per cent CO in atmospheric air

Equal parts of cell free medium from the responder and the medium pool from the stimulators were mixed and stored at 4 C for change of medium which was performed at day 2 4 and 6 with half of the culture volume

The cultures were harvested in duplicate with 24 hours intervals from day 2 to day 7 After 4 hours incubation with 0 2 μCi ^{14}C Thymidine (sp a 6 2 m Ci/mM The Radiochemical Centre Amersham England) the cells were collected on glass fibre filters and prepared for liquid scintillation counting as described previously (14)

All the samples were counted in a Packard Tri Carb Liquid Scintillation Counter and all results expressed as cpm – background per 1 × 10^6 responding lymphocytes of the initial cultures

From the material obtained from the family study 203 cultures harvested in

duplicate were analysed statistically The pooled estimate for the coefficient of variation was found to be 10 6 per cent If two clearly deviating results (coefficient of variation 50 8 and 45 2) probably due to technical errors are disregarded the coefficient of variation is reduced to 9 8 per cent

RESULTS

Table 1 and Fig 1 show the effect of the number of stimulating lymphocytes in four mixed cultures obtained from unrelated cell donors An increase in the number of stimulating lymphocytes resulted in increased response as measured during the entire culture period If the response as measured on day 3 or 4 was plotted against log (number of stimulating lymphocytes) linearity was obtained

TABLE 1

The Relationship between the Number of Stimulating Lymphocytes and the Responses Obtained in MLC between Unrelated Persons

Mixture	Number of stimulating lymphocytes	CPM Day of culture 2	3	4	5	6	7
B + Hm	3 00 × 10⁶	1093	5402	13164	24420	33484	20627
	2 00 × 10⁶	895	4749	11022	18046	21825	16300
	1 00 × 10⁶	593	3109	7355	14112	16231	9930
	0	141		444		665	
C + Sm	2 70 × 10⁶	175	1063	3858	13229	22090	16482
	1 35 × 10⁶	141	885	2646	6896	15357	11494
	0 67 × 10⁶	134	625	1960	4318	7197	6834
	0		124		265		449
T + Am	4 00 × 10⁶	755	2154	7849	20652	39851	31210
	2 00 × 10⁶	624	1985	6898	17318	33769	28873
	1 00 × 10⁶	478	1418	5376	13815	27815	19642
	0		120		307		422
E + Fm	4 00 × 10⁶	1408	6607	25231	40039	60635	55228
	2 00 × 10⁶	1001	5039	18205	30567	45537	19 72
	1 00 × 10⁶	762	3993	12421	21888	35311	11064
	0		124		204		542

For symbols see text

With a view to investigating the significance of the HL-A typing for the MLC-test three families R M and P were studied The results of the HL-A typing and the chromosome analyses appear from Table 2

It has not been possible in all family members to define all four HL-A antigens but as no blank chromosomes are believed to exist the undetectable assumed genes are marked with X Y and U

The HL-A typing permitted an unequivocal chromosome analysis in all 3 families the results of which are shown in small letters in column 4 Table 2

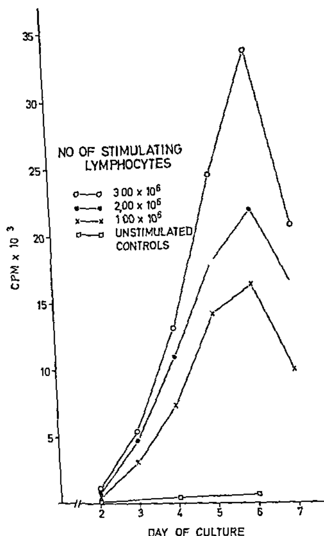


Fig 1
Unrelated mixture (B + Hm) containing increasing numbers of stimulating lymphocytes

The results of the MLC tests performed appear from Table 3 A B C etc refer to siblings within the same family X refers to the father Y to the mother A + Bm refers to a mixture of A leucocytes as responding cells and mitomycin treated lymphocytes from B as stimulating cells

In experiment 1 (Fig 2) A(ad) belonging in the R family is the responder Two HL-A identical siblings B(bc) and C(bc) both differing by two HL-A chromosomes from A(ad) and the sibling E(bd) differing only by one chromosome from A(ad) were stimulators As seen

TABLE 2

Results of ABO and HL-A Typing in 3 Families R M and P

Family		ABO A	ABO B	HL-A LA 1	2	Ba	3	HN	LA4	4 5	R	7	8	KNT12	KNHN	KNAL	RHUIS	Chromosomes	Genotypes	
R	1		+		+	−	−	−	−	−	−	−	−	+	−	+		ab	2 T12	/2 AI
	2	+		+	−	+	−	−	−	−	−	−	+	−	−	−		cd	1 8	/Ba
	f 3	+	+	+	+	−	−	−	−	−	−	−	+	+	−	−		ac	2 T12	/1 8
	(B) f 4		+	+	+	−	−	−	−	−	−	−	+	−	−	+		bc	2 AI	/1 8
	(E) m 5	+	+	+	+	−	−	−	−	−	−	−	+	+	−	−		ac	2 T12	/1 8
	(E) m 6		+	−	+	+	−	−	−	−	−	−	−	−	−	+		bd	2 AI	/Ba
	(A) m 7		+	−	+	+	−	−	−	−	−	−	−	+	−	−		ad	2 T12	/Ba
	(D) f 8		+	+	+	−	−	−	−	−	−	−	+	−	−			bc	2 (AI)	/1 8
	(C) f 9	−	+	+	+	−	−	−	−	−	−	−	+	−	−			bc	2 (AI)	/1 8
M	1	+	−	−	+	−	−	−	−	−	+	−	−	−	−			ab	2 X	/U R
	2	+	−	−	+	−	−	+	−	+	−	−	−	−	−			cd	2 Y	/HN 5
	m 3	+		−	+	−	−	−	−	−	−	−	−	−	−			ac	2 X	/2 Y
	(B) m 4			−	−	−	−	+	−	+	+	−	−	−	−			bd	U R	/HN
	f 5			−	−	−	−	+	−	+	+	−	−	−	−			bd	U R	/HN 5
	(D) f 6	+		−	+	−	−	−	−	−	−	−	−	−	−			ac	2 X	/2 Y
	(A) m 7	+	−	−	+	−	−	−	−	−	+	−	−	−	−			bc	1 R	/2 Y
	(C) f 8	+			+	−	−	+	−	+	−	−	−	−	−			ad	2 X	/HN 5
P	1	+	−	+	−	−	+	−	−	−	−	+	−	−	−		+	ab	1 7	/3 UIS
	2	+	−	−	+	+	−	−	−	−	−	−	−	+	−	−	−	cd	Ba T12	/2 X
	(C) m 3	+			+		+	−	−	−	−	−	−	−	−	−	+	bl	3 UPS	/2 X
	(A) f 4				+		+	−	−	−	−	−	−	−	−	−	+	bd	3 UIS	/2 X
	(D) f 5	+		+	+					−	−	+	−	−	−	−	−	al	1 7	/2 X
	(B) f 6		−	−	+	−	+		−	−	−	−	−	−	−	−	+	bd	3 UPS	/2 X
	(E) m 7	+		+	−	+	−	−	−	−	−	+	−	+	−	−	−	ae	1 7	/Ba T12

The ... chromosomes as derived from the HL-A typing and the HL-A genotyping. Symbols: The father is indicated with ... the children with the following number. The big letters in brackets refer to responders and stimulator in the MLC test shown in Table 3. Antigens 1 2 3 5 and 7 refer to the corresponding WHO accepted antigens HL-A 1 2 3 5 and 7. KNAL and RHUIS refer to incompletely investigated antigens presumably belonging to the 4 locus. The other specificities have been described previously.

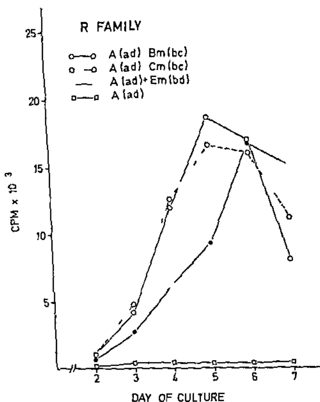


Fig 2

Experiment 1 The symbols represent mixtures and HL-A chromosomes See text

from the figure B(bc) and C(bc) measured from day 2 to day 5 gave clearly higher stimulation than I (bd) while there was no difference in the response at day 6

From experiment 2 it is seen that E(bd) differing by two chromosomes from the responder F(ac) when measured over the entire culture period gave clearly higher stimulation than A(ad) and D(bc) who share the chromosome a and c respectively with F(ac) The same pattern was found in experiment 3 in which the mother X(cd) clearly stimulated the father Y(ab) stronger than B(bc) who shared the chromosome b with the father

One additional experiment was performed in the R family In this experiment A(ad) during the entire culture period was clearly seen to stimulate D(bc) more strongly than E(bd) However this experiment was not included in the table as the mitomycin treated controls Am + Em showed significant activity In the remaining experiments the mitomycin treated controls have never exceeded the unstimulated controls of the responding cells

In the M family four mutually HL A different siblings were tested

TABLE 3

Results of MLC in the Three Families Studied

Family	Exp No	Mixture and HL-A chromosomes	Number of chromosomes stimulating	Stimulating HL-A antigens LA	Stimulating HL-A antigens 4	Number of antigens stimulating	CPM Day of culture 2	3	4	5	6	7
	1	A(ad) + Bm(bc)	2	1	8 AI	3	1012	4173	11915	18538	16772	8014
		A(ad) + Cm(bc)	2	1	8 AI	3	1075	4712	12501	16416	15906	11109
		A(ad) + Dm(bd)	1	0	AI	1	707	2739	6232	9279	16722	14892
		A(ad)					172	377	448	330	366	434
R	2	D(ac) + Bm(bd)	2	Ba	AI X	3	1001	7597	10641	29946	29798	8882
		D(ac) + Am(ad)	1	Ba	X	2	512	3127	6588	11719	8909	2977
		D(ac) + Dm(bc)	1	0	AI	1	534	3258	6423	11252	8736	4300
		D(ac)					187		350		267	
	3	Y(ab) + Xm(cd)	2	1 Ba	8 X	4	711	2419	4563	9005	18079	6728
		Y(ab) + Bm(bc)	1	1	8	2	444	1409	2025	2649	4440	3406
		Y(ab)						233		157		308
	4	A(bc) + Cm(ad)	2	11 X	5,X	3	410	1835	6059	11413	21573	18633
		A(bc) + Bm(bd)	1	11 N		2	427	1779	5608	13819	23758	23492
		A(bc) + Dm(ac)	1	0	X	1	456	1568	4679	11300	10873	13917
		A(bc)					66		238		339	
M		B(bd) + Dm(…)	2	22	XY	3	894	3569	12790	29167	35830	12789
		B(bd) + Am(…)	1		Y	2	598	2320	6920	15576	18136	13650
		B(bd) + Cm(ad)	1	2	X	-	400	1789	3860	11810	15395	9730
		B(bd)						275		557		849

	6	C(ad) + Am(bc)	2	U	R \	3	616	3393	17148	37353	47554	20960
		C(ad) + Dm(ac)	1	O	\	1	398	2149	9004	25982	41230	21984
		C(ad) + Bm(bd)	1	U	R	2	473	2598	12190	31462	46993	25557
		C(ad)						194		508		82
	7	D(ac) + Bm(bd)	2	U ILN	R 5	4	960	3792	14474	29927	41238	22291
		D(ac) + Cm(ad)	1	ILN	5	2	551	2315	6934	14000	18530	8804
		D(ac) + Am(bc)	1	1	R	2	648	2211	8412	17623	30094	13477
		D(ac)					210		404		351	
	8	A(bd) + Fm(ac)		1 Ba	7 T12	4	379	2161	9122	18504	27219	12802
		A(bd) + Dm(ad)	1	1	7	2	145	492	2190	5165	9969	5226
		A(bd)					61		273		255	
	9	B(bd) + Em(ac)	2	1 Ba	7 T12	4	758	4797	16553	31202	30028	12074
		B(bd) + Dm(ad)	1	1	7	2	201	1270	5410	10614	16423	7407
		B(bd)						332		598		1190
P	10	C(bd) + Fm(ac)	2	1 Ba	7 T12	4	691	3439	12375	23864	41043	26936
		C(bd) + Dm(ad)	1	1	7	2	221	1118	4405	9022	20938	17680
		C(bd) + Bm(bd)	0	–	–	0	138	223	447	600	814	653
		C(bd) + Am(bd)	0	–	–	0	192	205	516	580	1194	970
		C(bd)					99	214	377	400	456	661
	11	E(ac) + Am(bd)	2	2 3	UPS \	4	688	5404	20500	39548	41037	22649
		E(ac) + Bm(bd)	2	2 3	UPS \	4	669	4727	17517	37231	37620	20721
		E(ac) + Cm(bd)	2	2 3	UPS \	4	677	5970	19021	36630	28932	15443
		F(ac) + Dm(ad)	1	2	X	2	398	2396	9557	17572	19190	11152
		E(ac)					219		653		1154	

The number of stimulating HL A chromosomes and HL A antigens are derived from the data shown in Table 2

than in the two remaining cultures in which stimulation with one chromosome was performed

In columns 5 and 6 in Table 3 the specificity *and number of the* stimulating antigens are listed It appears that within the individual experiment a good correlation between the response and the number of stimulating antigens was obtained *Furthermore a comparison of* experiments shows that the correlation between the number of stimulating antigens and the provoked responses was poor

In the P family, three *HL-A identical siblings A(bd) B(bd) and* C(bd) were stimulated with the same two siblings differing with two E(ac) and one D(ad) chromosome from the responders (experiments 8 9, and 10) *As seen from the table the absolute magnitude of the* responses obtained was different but in all experiments E(ac) was found to give maximal stimulation In experiment 10 two siblings *both HL-A identical with the responder were included as stimulators* and as expected these were found to give only minimal stimulation

In experiment 11 E(ac) was stimulated by three HL-A identical siblings *who differed by two chromosomes from E(ac) As seen from Fig* 5 the three siblings gave approximately the same stimulation and they all stimulated stronger than D(ad) which has one chromosome in common with E(ac)

DISCUSSION

Quantitation of the lymphocyte response in vitro is most often carried out by measuring thymidine incorporation at a time at which the response is believed to be maximal *Festenstein* (8) in his work with the *mixed spleen cell reaction in mice* was the first *to point out that measuring* the response on a single day might be misleading due to the fact that strong combinations show a tendency to give an earlier peak than in *weaker combinations In the present investigation* peak response was found at day 5 or 6 and employing the same culture technique peak response has been observed on the 7th day (15) As seen from Tables 1 and 3 *in the single experiment* peak response was reached on the same day when either different numbers or different antigenic strengths of the stimulating cells were utilized However in experiments 1 9 and 11 a *tendency to an earlier peak* was seen when the stimulating cells had a greater antigenic strength Furthermore Festenstein has shown that peak response in strong combinations is followed by a pronounced *decline in thymidine incorporation* and as seen from Table 3 the same holds true for human cultures

Previous investigations have however raised doubts as to whether *peak response is a reliable* measure for the lymphocyte activation because maximal thymidine incorporation has been shown not only to be determined by the induced cell proliferation but also by the *inhibiting influence from suboptimal* culture conditions On the other hand the early response in mixed cultures measured on days 2 3 and 4 was

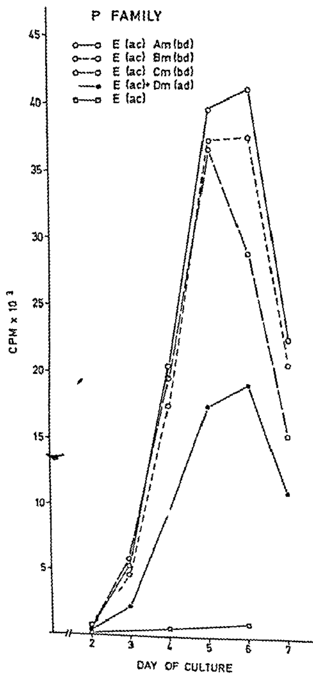


Fig 5

Experiment 11 The symbols represent mixtures and HL-A chromosomes See text

found to be influenced by culture conditions to a much smaller extent and was therefore from a technical point of view a better measure for the cellular reaction (14).

Furthermore it has been shown in tuberculin stimulated cultures that the optimal antigen concentration and the strongest hypersensitivity is best demonstrated by measuring the thymidine incorporation early in the culture period (14).

A similar observation has recently been reported by *Gurvich & Svet Moldavskaya* (9) who in morphologically evaluated smallpox stimulated cultures have demonstrated that the most hypersensitive persons are characterized by an early and intense blast transformation.

The main purpose of the present work has been to investigate whether the response in mixed cultures obtained from HL-A typed family members could be quantitated solely by measurements of the thymidine incorporation early in the culture period inasmuch as it was predicted that lymphocytes from persons differing by two HL-A chromosomes from the responder should give the maximal stimulation in the actual experiment.

In 10 experiments the results were completely in accordance with the predictions: as seen from Table 3 the two chromosome differing mixtures gave already from day 2 a higher response than the one chromosome differing mixtures and over the following 3 days this difference became more pronounced with an increasing statistical significance. In most cultures on day 3 and in all cultures on day 4 the difference was highly significant.

One experiment (No. 4) differs from the others since the response on stimulation with lymphocytes from the two chromosome differing sibling was not significantly higher than the response provoked by the two one chromosome differing siblings. Several reasons for this exception may be postulated.

Firstly, the responder's c chromosome contains the HL-A 2 gene which is also carried by the stimulating cells Cm(ad) and Dm(ac). This must be assumed to reduce the stimulation elicited in these two cultures. However it does not explain why the responses in the three cultures used in this experiment were of the same magnitude in spite of the fact that 3, 2 and 1 antigens respectively were stimulating in the three cultures. This could be explained by cross reactivity between the antigens belonging to the LA and 4 series respectively (10, 12). If HL-X has some antigen determinant in common with the LA antigen of the responder the influence of this antigen may be of minor importance and the stimulation will be determined mainly by the 4 antigens 5 and X. The responder is R and it is known from serological experiments that cross reactivity does exist between this antigen and HL-A 5 (12). This may indeed influence the results obtained and furthermore if also 5 and the hypothetical antigen X are cross reactive the results may be explained. This is of course hypothetical but it is in accord

ance with the serology of the HL-A system Technical difficulties thus need not be invoked in order to explain the result obtained in this experiment although this possibility cannot be excluded

Peak response did not always permit the same clear distinction between varying stimulations when compared with the initial response Fig 4 shows that one of the one chromosome differing siblings gave the same peak response as the two chromosome differing sibling while measurements made earlier in the culture period permitted a good distinction Fig 2 shows the same pattern One day after peak response two chromosome differing mixtures often showed a lower response than one chromosome differing mixtures due to the strong inhibition usually seen after a high peak response

In experiment 10 two siblings identical with the responder with respect to the HL-A determinants were included as stimulators As seen from Table 3 the activity in these two mixtures was in the same range as the unstimulated controls and from day 3 and onwards it was clearly lower than in the one chromosome differing mixture Further investigations are however necessary to evaluate the possibility of distinguishing between HL-A identical and low stimulated mixtures by measurements made only during the first four days of culture

In experiments Nos 1 and 11 stimulation was performed with 2 and 3 mutually HL-A identical siblings both groups differing from the responder by two chromosomes Figs 2 and 5 show the very close similarity in the responses obtained at days 2 3 and 4 which supports the suggestion that the early response is the best measure for the cellular reaction (14) Furthermore this finding is in accordance with the assumption that the reactivity in mixed cultures is largely determined by HL-A antigens

In experiments Nos 8 9 and 10 three HL-A identical siblings were stimulated by the same two HL-A non identical siblings differing from the responders by two and one chromosome respectively As seen from Table 3 the responses were in spite of the same antigenic stimulation rather variable However the lowest stimulated two chromosome mixture was clearly higher than the strongest stimulated one chromosome mixture

Comparing the results of experiments Nos 8 9 and 10 with experiments Nos 1 and 11 where the same person was stimulated to the same degree by lymphocytes from two and three different but mutually HL-A identical siblings it is tempting to conclude that the antigen content is very constant in HL-A identical siblings and thus genetically controlled mainly by the HL-A locus However the stimulation elicited is not only dependent on the antigen content of the stimulating cells but may be influenced by other variables These may be individual factors in the responding lymphocytes although influence of serum components cannot be excluded The experiments show that the varia

tions in the response of the individual lymphocyte culture cannot be caused by genetic information closely linked to the HL-A locus.

These results, if confirmed through further experiments, will be important for quantitation of MLC in relation to HL-A typing and clinical transplantations.

Table 1 and Fig. 1 show the correlation between the number of stimulating lymphocytes and the response in mixed cultures obtained from unrelated persons. Within the given limits an increased number of stimulating lymphocytes was always followed by an increased response, implying that maximal stimulation is not obtained by the routinely used number of stimulating lymphocytes of 1 $\times$ 10^6 per culture. *Albertini & Bach* (1) have reported cases of non-stimulation between HL-A non-identical siblings using the same number of stimulating and responding lymphocytes in the cultures. These investigators therefore stress the importance of making several experiments with increasing numbers of stimulating lymphocytes in the cultures, as stimulation is always obtained under these conditions. Employing the *present method, non-stimulation has never been seen in mixtures of* HL-A non-identical family members tested before transplantation (16). In a few weakly stimulated cultures, however, significant activity was not obtained until day 3 instead of as usual on day 2. Therefore it will probably be necessary to increase the number of stimulating lymphocytes in such weakly stimulated cultures in order to distinguish clearly between different degrees of stimulation within the first four days of culture.

In conclusion, the method described permits comparison between different donors' HL-A determined antigenic strength against the same recipient. However, it is not possible on the basis of the magnitude of a single response to obtain more than a very rough idea of the degree of incompatibility present.

SUMMARY

Previous studies on the *in vitro* stimulation of lymphocytes have suggested that the cellular thymidine incorporation early in the culture period was a better measure of the lymphocyte response than the later *occurring maximal thymidine incorporation.* The main purpose of this study has been to investigate whether this method would permit determination of the relative antigenic strength between members of an HL-A typed family.

Out of 11 experiments, all except one show that persons differing by two HL-A chromosomes provoke a higher stimulation, as measured during the first four days of culture, than persons differing by one chromosome. The maximal thymidine incorporation as measured on day 6 often gave erratic results.

It is concluded that the method represents an accurate means of distinguishing different lymphocyte donors' HL-A determined antigenic

strength in relation to a given responder within a single experiment However physiological variations among responders of the same HL A genotype cannot be excluded Conclusions on the antigenic strength based on the magnitude of a single response must therefore be regarded with caution

REFERENCES

1 *Albertini R J & Bach F H* Quantitative assay of antigenic disparity at HL-A —The major histocompatibility locus in man J Exp Med *128* 639–651 1968
2 *Amos D B & Bach F H* Phenotypic expressions of the major histocompatibility locus man (HL-A) Leukocyte antigens and mixed leukocyte culture reactivity J Exp Med *128* 623–637 1968
3 *Bain B Vas M & Lowenstein L* A reaction between leukocytes in mixed peripheral blood cultures Federation Proc 22 428 1963
4 *Bain B Vas M & Lowenstein L* The development of large immature mono nuclear cells in mixed leukocyte cultures Blood 23 108–115 1964
5 *Bach F H & Voynow N K* One way stimulation in mixed leukocyte cultures Science *153* 545–547 1966
6 *Bach F H Albertini R J Klostermann H & Day E* Histocompatibility Testing Munksgaard Copenhagen 1967 p 55
7 *Ceppellini R Curtoni E S Mattiuz P L Miggiano V Scudeller G & Serra A* Histocompatibility Testing Munksgaard Copenhagen 1967 p 149
8 *Festenstein H* Antigenic strength investigated by mixed cultures of allogeneic mouse spleen cells Ann N Y Acad Sci *129* 567–572 1966
9 *Gurvich E B & Svet Moldavskaya J A* Transformation of human blood lymphocytes under the influence of small pox antigen Nature *220* 1050–1051 1968
10 *Kissmeyer Nielsen F Svejgaard A & Hauge M* Genetics on the human HL-A transplantation system Nature *219* 1116–1119 1968
11 *Kissmeyer Nielsen F & Kjerbye K E* Histocompatibility Testing Munksgaard Copenhagen 1967 p 381
12 *Kissmeyer Nielsen F* Unpublished work
13 *Svejgaard A & Kissmeyer Nielsen F* Complement fixing platelet iso anti bodies I A quantitative technique for their detection Vox Sang *14* 106–118 1968
14 *Sørensen S F Andersen V & Giese J* Studies on the quantitation of lympho cyte response in vitro Acta path microbiol scand In press (1969)
15 *Sørensen S F* Unpublished work

Acta path microbiol scand 77 134-142 1969

The Department of Microbiology and Immunology Veterinary College of Norway
Oslo Norway Head Professor dr med vet Olav Sandvik

QUANTITATIVE ASSAY OF A SINGLE PROTEOLYTIC ENZYME IN A CRUDE MIXTURE OF BACTERIAL PROTEINASES

By

HANS KOLBEIN DAHLE

Received 20 iii 69

The extracellular microbial proteinases are responsible for an important part of the biochemical activity in many ecosystems. They may represent a complex mixture which makes the separate identification and titration of the various enzymes very problematic. The different proteinases may be produced partly by a single organism (*Sandvik* 1962) and partly by a more or less complex flora of micro organisms. It is however of great interest to be able to study the various proteinases separately in pure cultures as well as in complex ecosystems such as food.

An agar plate method has been described for quantitative determination of a predominating or single proteinase in a crude environment (*Sandvik* 1962). By this method the so called Casein Precipitating Enzymes (abr CP enzymes) give characteristic white precipitation zones when acting on sodium caseinate in an agar medium. Serial 2 fold dilutions of the enzyme containing material were made and a definite amount from each dilution was transfered to corresponding wells in the substrate plate. The titre was estimated on the basis of the zone diameters after incubation.

At present however no suitable procedure for large scale identification and titration of the individual proteinases in a crude mixture is available. The main problem is to be able to inactivate all the enzymes except the one selected for determination. In the present work a procedure based on the selective neutralization of the enzymes not wanted has been used for this purpose. This neutralization was obtained by adding specific antiproteinases to the substrate plate.

MATERIALS AND METHODS

Strains The strains used were from The Culture Collection at the Department of Microbiology and Immunology Veterinary College of Norway (NVH). *Aeromonas salmonicida* was obtained from the American Type Culture Collection Rockville Maryland U S A (ATCC). The strains are listed in Tables 1 to 3.

Enzymes The various proteinases were usually produced by growing the organism

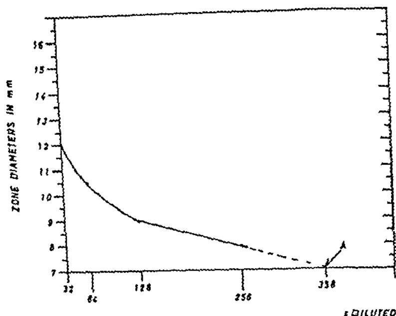


Fig 1

Determination of diffusion units Zone diameters plotted against degree of dilution for CP enzymes produced by *Bacillus cereus* (NVH 32?)

on semi solid skim milk agar (nutrient broth 40 per cent nutrient agar 4? per cent autoclaved skim milk, 18 per cent) in Roux bottles for 3–5 days at 37 C. *Ae salmonicida* was grown at 30 C The harvesting of cultures and the concentration and purification procedure have been described (*Sandvik* 1962)

Sera. Antiproteinases were produced in rabbits The concentrated enzyme preparations were mixed with equal amounts of Freund s complete adjuvant (Difco) and injected subcutaneously in amounts of 2 0–4 0 ml at 6 day intervals One week after the third injection the blood sera were tested for specific anti enzymes

In addition to the specific anti enzymes these sera contained normal inhibitors against microbial proteinases and it has been previously demonstrated that the inhibitors were in the α and β globulin fractions (*Sandvik* 196?) Preliminary investigations showed that the normal inhibitors against proteinases interfered with the specific anti enzymes contained in the γ globulin fraction In order to exclude these inhibitors serum proteins were separated by paper electrophoresis after which the γ globulin fraction was cut off and eluated with water Antisera without normal inhibitors were thus obtained but the method was too cumbersome for the production of sufficiently large quantities for the serial experiments The sera were therefore precipitated in salt solutions decreasing from 18 to 12 per cent Na_2SO_4 in order to isolate the γ globulin fractions (*Stelos* 19(7) Paper electrophoresis (*Aronsson & Grönwall* 1957) and disk electrophoresis (*Davis* 1964) were used to test the fractions for α β and γ globulins but only γ globulins could be demonstrated

Substrate and incubation Sodium caseinate in agar plates was used as indicator medium for the CP test It was prepared as follows Bacto agar (Difco 0140 01) 1 40 per cent sodium caseinate (added as 4 per cent solution of pH 6 2) 1 00 per cent thiomersal 0 01 per cent $MgCl_2$ (added as 10 0 per cent w v solution) 0 004 M in water The pH was adjusted to 6 ? The total volume of substrate used in each plate was 44 ml which gave a 2 mm thick agar layer The incubation was always 12 hours at 37 C

Titrations After trial titrations standard solutions of each crude enzyme solution and of enzymes in mixtures were prepared The dominating enzymes in a mixture containing more than one proteinase could be titrated by transfering ?5 µl

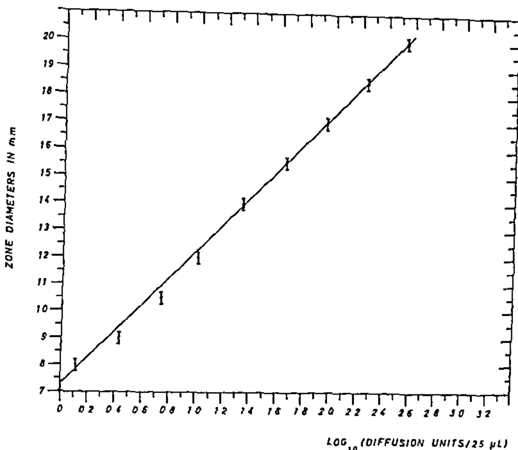


Fig 2
Logarithmic standard curve for a CP enzyme produced by *Bacillus cereus* (ref Table 1 and Fig 1)

from serial 2 fold dilutions of enzymes to corresponding circular wells of 7 mm diameter in the substrate plate After incubation the diameters of the precipitation zones were measured to the nearest 0 5 mm The determination of diffusion units given by *Sandvik* (1962) was used and the number of diffusion units in 25 µl was estimated from curves of the type shown in Fig 1 where the measured diam ters were plotted against degree of dilution The curves were then extrapolated to the abcissa where the point A (Fig 1) indicates the number of diffusion units in µl As a modification from the method given by *Sandvik* a graphical adjustment of the number of diffusion units was introduced Thus the logarithmic standard curves were drawn as the best straight line after marking the uncertainty with re pect to zone diameters (Fig 2) This straight line was not predetermined to go thr ugh the origo The corrected number of diffusi n units was obtained by going fr m the point on the ordinate axis corresponding to the actual z ne diameter t the *standard curves*

Replicate titrations were also carried out after adding t each diluti n tube an excess of pecific anti enzyme against certain enzymes in the mixture

Titrations of the specific antisera were carried out in 2 fold serial dil ti ns Standard amounts of the homologous enzyme were added to each tube in the dilution series in order to estimate the smallest v lume of specific antiserum that had to be incorporated into the sodium caseinate agar to neutralize the eff ct of the enzyme As a rule a fourfold concentration of the estimated titre was u ed in the agar medium to secure a complete inactivation of the enzymes other than the particular one selected for examination

RESULTS

The enzyme concentrations of the standard solutions are shown in Table 1 These solutions include proteinases produced by a single organism as well as 2 mixtures of enzymes produced by 2 different bacteria (Fig 3) Table 1 shows also that the CP activity was completely neutralized by adding excess of homologous anti enzyme to the dilution series before application of the mixture to the substrate plate On the other hand the mixture containing anti enzyme against only one of the proteinases was only partly neutralized With *Ps aeruginosa* a residual CP activity was always present in spite of excess of the corresponding antiserum Likewise addition of heterologous antiserum in excess did not seem to influence the enzyme titre

A fairly low enzyme concentration (about 400 diffusion units in 25 μl) was generally used for determining the necessary amount of specific γ globulin to be added to the plates in order to obtain complete neutralization of the unwanted enzymes The concentrations (four times the smallest amount) in ml of specific γ globulin per ml of substrate are presented in the right hand column in Table 2 but experiments have shown that addition of a large excess of anti enzyme does not interfere with the results

Substrate plates containing anti enzymes were used for titrations of proteinases produced by various bacteria (Fig 3) Precipitation zones were not observed when the enzyme homologous to the anti enzyme incorporated in the plates was tested On the other hand the titre of

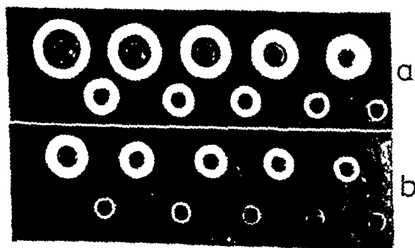


Fig 3

Titrations of CP activity a Precipitation zones caused by a mixture of proteinases in sodium caseinate agar (serial 2 fold dilution) b Replicate titration on a sodium caseinate agar plate with anti enzymes against one of the two proteinases incorporated

TABLE 1

Comparison of the Enzyme Activity of Standard Solutions with and without Addition of an Anti-Enzyme in Excess

Enzyme-anti-enzyme mixture: Enzyme produced by	Excess anti-enzyme against	Dilution of enzymes: 1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	Diffusion unites in 25 μl	Notes
Aeromonas salmonicida (ATCC 14174)	None	20	18.5	17	15.5	14	13	12	11	10	9	8	–	1690	
–	*Aeromonas salmonicida*		–	–	–	–	–	–	–	–	–	–	–		Completely inhibited
Bacillus cereus (NVII 322)	None	20	18	17	15.5	14	12	10.5	9	8	–	–	–	350	
	Bacillus cereus		–	–	–	–	–	–	–	–	–	–	–		– –
Pseudomonas fluorescens (NVII 656)	None	17.5	16	15	14.5	13	12	10	9	8	–	–	–	300	
	Pseudomonas fluorescens		–	–	–	–	–	–	–	–	–	–	–		– –
Pseudomonas aeruginosa (NVII 150)	None	22	21	18.5	17	16	15	13.5	12	10.5	9	8	–	1450	
–	*Pseudomonas aeruginosa*		12	10	9	–	–	–	–	–	–	–	–		Partly inhibited
Pseudomonas aeruginosa + *Bacillus cereus*	None	22	20	18	17.5	16.5	15.5	14	13	12	10.5	9	7.5	3470	
	Bacillus cereus		18	16	14	12	–	–	–	–	–	–	–		– –
Pseudomonas fluorescens + *Aeromonas salmonicida*	None	18.5	17	16	15	14	13.5	12.5	11.5	10.5	10	9	7.5	2630	
" *Bacillus cereus*	*Aeromonas salmonicida* *Pseudomonas fluorescens*		14	13	12	10.5	9	8	–	–	–	–	–		– –
			18.5	17	15.5	13.5	12	10.5	8.5	8	–	–	–		*Control*

The proteinases are indicated by the organism by which they are produced.
The figures [illegible] in millimeters.

TABLE 2

Determination of the Minimum Amount of Specific Anti Enzyme which Should be Added to Substrate Plates in Order to Completely Neutralize the Unwanted Enzymes

Enzyme produced by	Specific anti enzyme added	Dilutions purified γ globulins§ 1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024	Amount of purified γ globulin to be added per ml substrate
Ae salmonicida	*Ae salmonicida*	–	–	–	–	–	–	10 5†	11	11	11	0 064 ml
Bac cereus	*Bac cereus*	–	–	–	–	–	–	–	–	–	10	0 008 ml
Ps fluorescens	*Ps fluorescens*	–	–	–	–	–	–	9	9	9	9	0 004 ml
Ps aeruginosa	*Ps aeruginosa*	–	–	–	–	–	–	–	10	12 5	12 5	0 032 ml
Ps aeruginosa	*Bac cereus*	13 5	13 5	13 5	13 5	13 5	13 5	13 5	13 5	13 5	13 5	Control

The specific anti enzymes are indicated by the organism by which the homologous proteinases are produced

§ The purified γ globulins were first diluted to the original volume of the sera

† The figures given are the zone diameters in millimetres

TABLE 3

Titrations of CP Enzymes on Ordinary Substrate Plates and on Plates with Added Specific Anti-Enzyme

Specific anti-enzymes added	Enzymes produced by	Dilutions of enzymes											
		1	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024	1 2048
None	*Ae. salmonicida*	20§	18.5	17	15.5	14	13	12	11	10	9	8	–
	Ps. fluorescens	17.5	16	15	14.5	13	12	10	9	8	–	–	–
	Ps. aeruginosa	22	21	18.5	17	16	15	13.5	12	10.5	9	8	–
	Bac. cereus	20	18.5	17	15.5	14	12	10.5	9	8	–	–	–
Ae. salmonicida	*Ae. salmonicida*	–	–	–	–	–	–	–	–	–	–	–	–
	Ps. aeruginosa	22	21	18.5	17	16	15	13.5	12	10	9	8	–
	Bac. cereus	20	18.5	17	15.5	13	12	10.5	9	8	–	–	–
Ps. aeruginosa	*Ps. aeruginosa*	1[illegible]	10	9	–	–	–	–	–	–	–	–	–
	Ae. salmonicida	20	19.5	17	15.5	14	13	12	11	10.5	9	8	–
	Bac. cereus	20	18.5	17	15.5	13.5	12	10.5	9	8	–	–	–
Ae. salmonicida + *Ps. aeruginosa*	*Ae. salmonicida*	–	–	–	–	–	–	–	–	–	–	–	–
	Ps. fluorescens	–	–	–	–	–	–	–	–	–	–	–	–
	Bac. cereus	20	18.5	17.5	15.5	13	12	10.5	9.5	8	–	–	–

The specific anti-enzymes are indicated by the organism by which the homologous proteinase are produced

§ The figures given are the zone diameters in millimetres

heterologous proteinases was the same as on ordinary substrate plates without anti enzymes (Table 3) An exception however was observed with regard to *Ps aeruginosa*

DISCUSSION

The experiments indicate that it is possible to perform fractional titrations of an artificial mixture of proteinases provided the necessary specific anti enzymes are available

The enzymes not being titrated can be successively neutralized leaving only one proteinase in the mixture active and the residual activity after such a neutralization will be caused by an enzyme which does not correspond to the neutralizing anti enzymes In the case of the mixture of proteinases produced by *Ps fluorescens* and *Ae salmonicida* when neutralized by the anti enzyme corresponding to *Ae salmonicida* (Table 1) the residual activity will be due to the enzyme from *Ps fluorescens* Mixtures of proteinases produced by only one organism seem also to behave similarly In this connection attention is drawn to the residual CP activity which can be demonstrated for crude proteinase concentrates from *Ps aeruginosa* when titrated in the presence of an excess of corresponding anti enzyme (Tables 1 and 3) In spite of the fact that this residual activity was only a small fraction of the total CP activity in the crude preparation it was not influenced by increasing amounts of *Ps aeruginosa* anti enzyme This means that the enzyme mixture contained at least one enzyme fraction which had no corresponding anti enzyme in that antiserum The enzyme in question was therefore not intact with respect to antigenicity in the enzyme adjuvant mixture which was used in the immunization of rabbits for production of the antiserum Therefore the residual enzyme fraction could be titrated by neutralization of the main component by *Ps aeruginosa* anti enzyme The experiments indicate also that the residual activity is not affected even in large excess of the neutralizing anti enzyme

The circumstances mentioned above can be of taxonomical value when seen in connection with the enzymoserological classification of bacteria which has been described (*Sandvik* 1962) It seems possible to subdivide the groups determined by Sandvik s method when the organisms to be classified produce more than one proteinase that can be studied separately

As rabbit serum contains normal inhibitors against microbial proteinases it is necessary to remove the α and β globulin fractions in order to isolate γ globulin fractions free from natural inhibitors which are suitable for fractional titrations of proteinases The results show that the described method for the production of such γ globulins is suited to this end Evidence for this is given nethermost in Table 1 where it is shown that large excesses of heterologous anti enzyme do not affect the enzyme titre of the selected enzyme

Neutralization was carried out in tubes as well as directly on the substrate plates but whether the activities of the anti enzymes were of the same order or lower when acting in the presence or absence of the substrate (*Cinader* 1963) *was not determined* The investigations show however that the CP reaction of the proteinases can be inhibited when the enzymes are added to an anti enzyme substrate mixture and also when enzyme anti enzyme mixtures are added to the substrate

The *titration of the main component of CP enzymes* has been used in food hygiene to estimate the concentration of proteolytic enzymes in the products (*Rossebø* 1968) In the light of the results presented it should be possible to chart complexes of various enzymes in such ecosystems as food It is also clear that the enzymological examination of food products will be of considerable importance for the understanding of the microflora and for the appraisement of the durability of the products

In conclusion, the results indicate that the methods presented can be used for the study of a single proteinase in a mixture of proteinases

SUMMARY

A particular proteinase in a mixture can be studied by the successive addition of specific homologous antiproteinases in order to inhibit the other proteinases Only purified γ globulin fractions of immune rabbit sera were used because of the content of normal inhibitors against microbial proteinases in untreated sera It was shown that even large excesses of immune γ globulin did not interfere with the titrations of the selected enzymes

The possibility of using the method described in studying bacterial spoilage in ecosystems such as food has been discussed

REFERENCES

Aronsson T & Gronwall A Improved separation of serum protein in paper electrophoresis—a new electrophoresis buffer Scand *J Clin Lab Invest 9* 338 341 1957

Cinader B Antibodies against enzymes Ann N Y Acad *Sci 103* 495–548 1963

Davis B J Disc electrophoresis—*II Method and application to human serum pro*teints Ann N Y Acad Sci *121* 404–427 1964

Rossebø I Personal communication 1968

Sandvik O Studies on casein precipitating enzymes of aerobic and facultatively anaerobic bacteria 116 pp Veterinary College of Norway Oslo 1962

Stelos P Salt fractionation Handbook of Experimental *Immunology* Blackwell scientific publications Oxford and Edinburgh *3 9 1967*

Acta path microbiol scand 77 143-148 1969

The Department of Microbiology and Immunology Veterinary College of Norway Oslo Norway Head Professor dr med vet Olav Sandvik

ENZYMOSEROLOGICAL SEPARATION OF BACTERIAL PROTEINASES

By

HANS KOLBEIN DAHLE

Received 11 iv 69

Serological examination of extracellular bacterial proteinases by means of an immunoelectrophoretic method has been described (*Sandvik* 1962) By this method specific antiproteinases in immune rabbit sera were separated from normal proteinase inhibitors by paper electrophoresis after which their antiproteolytic effect was demonstrated by inhibiting the specific enzyme from precipitating sodium caseinate in an agar medium Many serologically different enzymes were produced by the organisms examined and most of the enzymes were specific in respect of species Cross reactions between species and genera were exceptional Thus the method can be used in identifying various organisms

While working with serological differentiation (*Sandvik & Hagen* 1968) and identification (*Dahle & Nordstoga* 1968) of aeromonads an unexpected picture appeared on the substrate plates after developing the immunoelectrophoretic patterns (*Sandvik* 1962) The enzyme produced by *Aeromonas salmonicida* was inhibited in the expected way by homologous antiserum while the enzymes produced by *Aeromonas liquefaciens* were only partly inhibited by its corresponding antiserum It was proposed that the picture regarding *Ae liquefaciens* was due to 2 enzymes A and B produced by the organism and that the antiserum was only active against A

It has been demonstrated that one particular organism can produce more than one proteinase (*Sandvik* 1962) and it was possible to prepare antienzymes against isolated fractions of the enzyme complex As the observations mentioned for *Aeromonas* species were never noticed with other organisms the question arose if the hypothesis of 2 enzymes (A and B) was correct regarding the immunoelectrophoretic patterns of *Ae liquefaciens* or if the phenomenon was due to some other reason

The present work deals with enzymoserological methods for the examination and identification of the single proteinases in pure bacterial cultures containing a mixture of such enzymes

Neutralization was carried out in tubes as well as directly on the substrate plates but whether the activities of the anti enzymes were of the same order or lower when acting in the presence or absence of the substrate (*Cinader* 1963) *was not determined* The investigations show however that the CP reaction of the proteinases can be inhibited when the enzymes are added to an anti enzyme substrate mixture and also when enzyme anti enzyme mixtures are added to the substrate

The titration of the main component of CP enzymes has been used in food hygiene to estimate the concentration of proteolytic enzymes in the products (*Rossebø* 1968) In the light of the results presented it should be possible to chart complexes of various enzymes in such ecosystems as food It is also clear that the enzymological examination of food products will be of considerable importance for the understanding of the microflora and for the appraisement of the durability of the products

In conclusion the results indicate that the methods presented can be used for the study of a single proteinase in a mixture of proteinases

SUMMARY

A particular proteinase in a mixture can be studied by the successive addition of specific homologous antiproteinases in order to inhibit the other proteinases Only purified γ globulin fractions of immune rabbit sera were used because of the content of normal inhibitors against microbial proteinases in untreated sera It was shown that even large excesses of immune γ globulin did not interfere with the titrations of the selected enzymes

The possibility of using the method described in studying bacterial spoilage in ecosystems such as food has been discussed

REFERENCES

Aronsson T & *Gronwall A* Improved separation of serum protein in paper electrophoresis—a new electrophoresis buffer Scand J Clin Lab Invest 9 338 341 1957

Cinander B Antibodies against enzymes Ann N Y Acad Sci *103* 49–548 1963

Davis B J Disc electrophoresis—II Method and application to human serum proteins Ann N Y Acad Sci *121* 404–427 1964

Rossebø L Personal communication 1968

Sandvik O Studies on casein precipitating enzymes of aerobic and facultatively anaerobic bacteria 116 pp Veterinary College of Norway *Oslo 196*[illegible]

Stelos P Salt fractionation Handbook of Experimental Immunology Blackwell scientific publications Oxford and Edinburgh 3–9 1967

homologous type of reaction with proteinases produced by *Ae salmonicida* grown in all the media in question (Table 1)

TABLE 1
Proteinases Produced by Ae liquefaciens *and* Ae salmonicida *when Grown in 3 Different Media Tested with Antiproteinases*

Proteinases produced by	Inhibition reaction when testing the proteinases with antienzymes against		
	Ae liquefaciens (SMA)	*Ae liquefaciens* (NG)	*Ae salmonicida* (SMA)
Ae liquefaciens SMA and L	A +++ (Fig 1 b) B —	— (Fig 1 c)	—
Ae liquefaciens P	A +++ B —	—	—
Ae liquefaciens NG	—	+++	+++
Ae salmonicida SMA and L	—	+++ (Fig 1 a)	+++
Ae salmonicida P	—	+++	+++
Ae salmonicida NG	—	+++	+++

+++ Inhibitory effect equal to homologous reaction A and B designate 2 different proteinases
SMA = enzymes produced in semi solid skim milk agar
P = enzymes produced in pepton water
NG = enzymes produced in nutrient gelatine
L = enzymes produced in litmus milk

In order to demonstrate a possible close relationship or identity between (1) the *Ae liquefaciens* NG enzyme and (2) the B fraction of the *Ae liquefacines* SMA enzyme complex substrate plates were prepared with *Ae liquefaciens* NG antiproteinase incorporated (*Dahle* in press) On plates of this type all the activity of enzyme fraction B was inhibited and the residual activity which was due to enzyme A could therefore be used in the identification test with the *Ae liquefaciens* SMA antienzyme The picture on such plates after developing *Ae liquefaciens* SMA antiserum with supernatant from cultures of *Ae liquefaciens* grown in litmus milk or pepton water indicated a homologous type of reaction Likewise enzyme A could be titrated as described (*Dahle* in press)

Comparable investigations were carried out with proteinases produced by *Ps aeruginosa* and the corresponding antiserum Also in this case 2 serologically different enzymes could be demonstrated by enzymoserological separation

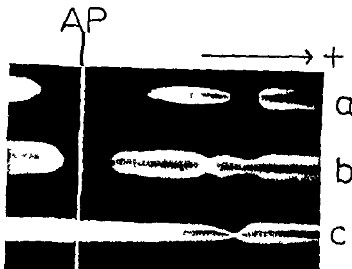


Fig 1

Electrophoretic patterns for antisera against casein precipitating enzymes of aeromonads transferred to sodium caseinate agar Developments were performed with 3 different enzyme solutions

a homologous reaction when *Ae salmonicida* SMA antiserum is developed with *Ae salmonicida* SMA enzyme

b *Ae liquefaciens* SMA antiserum developed with *Ae liquefaciens* SMA enzymes Enzyme A is specifically inhibited while enzyme B is not affected

c *Ae liquefaciens* SMA antienzyme developed with *Ae liquefaciens* NC enzyme

The specific antibodies are localized in the area of the line of application (AP) The normal serum inhibitors can be seen to the right of this line The electrophoresis was carried out in 0 05 M phosphate buffer pH 6 2 for 18 hours at 120 v

DISCUSSION

With the immunoelectrophoretic method described by *Sandvik* (1962) it is possible to perform enzymoserological identification of single isolated enzymes or the total enzyme complex produced by a particular organism The present procedure may open possibilities for the identification of separate proteinases in complexes of enzymes directly without isolation of the individual proteinases by for example ion exchange chromatography By this procedure which is called *enzymo serological separation,* specific antienzymes against all the proteinases except the particular one to be studied are added to the substrate plate In the present work this method is used for studying proteinases produced by *Aeromonas* and *Pseudomonas* species

Ae liquefaciens produced in all media used a proteinase B identical with or closely related to the single one produced by *Ae salmonicida* In addition a proteinase A specific for *Ae liquefaciens* was demonstrated in pepton water and milk media but not in nutrient gelatin

The experiments performed with 3 different media indicated that certain factors in the media may induce the production of particular enzymes or inhibit the activity of others This is important from a taxonomical point of view because the composition of the media has to be taken into consideration when using the method for enzymoserological classification of microbes Furthermore a systematic analysis of the various enzymes produced *under standard conditions* will give increasing possibilities for type differentiation of the organism in question

From Table 1 it is seen that the proteinases produced by *Ae liquefaciens* grown in pepton water or litmus milk give no inhibition reaction with the *Ae liquefaciens* NG antiserum or the *Ae salmonicida* SMA antiserum These observations are probably due to the fact that the sera used contained antibodies against proteinase B only and not against the dominating proteinase A in the cultures Thus a homologous type of reaction was not observed

Both *Ae liquefaciens* SMA antiserum and *Ps aeruginosa* SMA antiserum seemed to contain antienzymes against only one component in the corresponding proteinase mixtures This is remarkable because both components existed in the antigen used for immunization The explanation is probably that one particular proteinase dominated in relation to the other when the cultures were harvested or that only one enzyme was antigenically active at the time of immunization As the CP reaction is extremely sensitive it may be possible that some proteinases demonstrated may have been present in amounts too small to induce antibody production This condition can also be expected in other systems and the sensitivity of the identification test for the antigens must also be considered in this connection

In conclusion the *enzymoserological separation* technique used seems to be valuable for studying separate proteinases in mixtures and for further extension of a bacterial taxonomical system based on antigenic properties of the proteinases produced by the organisms This requires however that a collection of the necessary antienzymes is available

SUMMARY

A procedure called *enzymoserological separation* is used for identification of bacterial proteinases produced by *Aeromonas* and *Pseudomonas* species It is shown that *Ae liquefaciens* produced 2 proteinases (A and B) when grown in litmus milk or pepton water but only one (B) when grown in nutrient gelatin The proteinase B is shown to be enzymoserologically identical with that produced by *Ae salmonicida*

It is demonstrated that the number of different proteinases produced by the organism is related to composition of the culture medium used This fact is important regarding the method of classifying bacteria on the basis of serological differentiation of their extracellular pro

teinases Problems in connection with general application of this method for taxonomical purposes are discussed

REFERENCES

Dahle H K Quantitative assay of a single proteolytic enzyme in a crude mixture of bacterial proteinases Acta path microbiol scand 77 134–142 1969

Dahle H K & Nordstoga K Identification of aeromonads in furred animals Acta vet scand 9 65–70 1968

Sandvik O Studies on casein precipitating enzymes of aerobic and facultatively anaerobic bacteria 116 pp Thesis Veterinary College of Norway Oslo Norway 1962

Sandvik O Identification of moulds by serologic differentiation of their proteolytical enzymes Acta path microbiol scand 71 333–338 1967

Sandvik O & Hagen O Serological studies on proteinases produced by *Aeromonas salmonicida* and other aeromonads Acta vet scand 9 1–9 1968

Acta path microbiol scand 77 149-161 1969

Department of Microbiology The Gade Institute and Medical Department A
School of Medicine University of Bergen Bergen Norway

BACTERIAL COUNTS IN URINE

2 A Comparison of Different Methods

By

J Haugen O Strom and B Østervold

Received 12 iii 69

Bacterial counts in urine may be performed by different methods Several modifications of the classic pour plate method have been suggested Another method used is surface spreading on solid media of a diluted or undiluted urine (3 6)

Both the preparation of pour plates and the dilution of urine are time-consuming and involve a risk of introducing technical errors The transfer to and spreading of urine on solid media by calibrated loops as proposed by *Hoeprich* (7) is thought to be a more convenient method for common clinical use

The main objection to the loop method has been the variation in the volumes of urine transferred by loops to the media We (4) have previously shown that with the technique used by us the variation in volumes transferred was relatively small and should therefore not give grounds for excluding the method Other factors such as the composition of the media and differences in the culture technique may influence the bacterial counts obtained

The bacterial counts obtained by the loop method as used by us are compared to the counts obtained by two other commonly used methods the pour plate method of *Kass* (8) and of *Merrit* (11) respectively

MATERIAL AND METHODS

Urine Urine specimens containing a single strain of bacteria were chosen from those submitted to the bacteriological laboratory for examination The specimens were kept in glass test tubes and consisted of 20-25 ml of urine

Loops Two bacteriological loops calibrated to deliver 0 01 and 0 001 ml urine respectively were used (7) The mean volume of physiological urine delivered by each loop to the media was determined (4) and a correction factor (c f) was calculated for each loop

Pipettes Serological pipettes (Silberbrand) of 1 0 (graded 1/100) and 10 0 ml (graded 1/10) were used

Dishes Sterile plain Petri dishes diameter 9 and depth 1 5 cm were used for all cultures

---

Request for reprints should be addressed to Dr *J Haugen* Haukeland hospital Bergen Norway

Quantitative cultures. Loop method. With each loop one loopful of urine was transferred to and spread on the medium by the streaking technique recommended (9) on a half plate of blood agar and a half plate of lactose agar respectively.

Kass method. 0.5 ml of urine was pipetted into one Petri dish and 0.1 ml of a dilution of 0.1 ml urine and 9.9 ml broth into another dish. The plates were then poured melted cooled agar (8) being used. *Merrit's method.* 1.0 ml of urine diluted with saline 1:10 and 1:1000 respectively was pipetted into each Petri dish and 9.0 ml of melted cooled tryptic digest agar (Difco) was added to each dish (11).

Incubation. All cultures were incubated for 16–18 hrs at 37 C.

Bacterial counting. The number of colonies grown was taken as the original number of bacteria in the urine volume transferred. The number of bacteria or the bacterial count per ml of urine was calculated for each method as described below.

Loop method. When a colony number less than 100 was obtained by the 0.01 ml loop this number × 100 × c.f. was stated. Otherwise the number given by the 0.001 ml loop × 1000 × c.f. was stated. For Gram negative bacteria the mean number of colonies on the blood agar and the lactose agar plates was used. *Merrit's method.* When a colony number less than 500 was obtained from the 1:10 dilution this number × 10 was stated. Otherwise the number of colonies given by the 1:1000 dilution × 1000 was stated. *Kass method.* As described for Merrit's method with the difference that the colony numbers were multiplied by 2 and 1000 respectively.

Identification. From the quantitative cultures the colonies were isolated and the bacteria identified by their staining, culture, fermentation and other biochemical properties according to commonly used methods.

Statistical methods. The mean, standard deviation (S.D.) and coefficient of variation (C.V.) were calculated by the commonly used methods. The difference between means was calculated by the t test. A 2 per cent (0.02) level of significance was used unless otherwise stated.

TABLE 1

Mean Counts of Ten Subsamples, Standard Deviation (S.D.) and Coefficient of Variation (C.V.) of Bacteria in Urines Examined by Three Different Methods of Urine Culture

Urine no.	Bacteria	Loop method Count × 10³	Loop method S.D. × 10³	Loop method C.V. %	Kass method Count × 10³	Kass method S.D. × 10³	Kass method C.V. %	Merrit's method Count × 10³	Merrit's method S.D. × 10³	Merrit's method C.V. %
1	E. coli	4.23	0.36	8.5	9.50	1.84	19.4	9.30	[illegible]	8?.7
2		12.10	1.63	14.?	23.00	6.10	26.3	21.00	3.80	18.1
3		34.00	[illegible]	9.6	43.80	8.75	20.0	42.90	5.84	13.6
4		37.00	4.80	13.0	38.90	3.90	10.0	41.10	3.60	8.8
5		47.34	6.07	12.8	97.20	9.93	10.2	73.50	8.06	11.0
6	"	62.60	5.68	9.2	84.50	4.60	5.4	82.70	7.66	[illegible]
7	Klebsiella	2.20	0.52	24.6	2.70	1.70	63.1	4.40	1.27	28.9
8	"	9.65	0.92	9.6	8.60	2.32	27.0	5.40	[illegible]	49.2
9	"	10.20	1.03	10.1	18.00	2.49	13.8	14.00	[illegible]	31
10		21.64	2.56	11.8	28.60	4.06	14.2	27.70	[illegible]	20.6
11		28.35	2.21	7.8	36.50	[illegible]	17.9	33.60	7.3?	14.9
12		47.20	5.00	10.6	58.40	6.85	11.7	63.20	7.36	11.6
13	Str. faec.	34.10	4.34	12.7	25.70	4.19	16.3	31.10	3.91	12.6
14		60.50	[illegible]	12.5	47.70	4.57	9.6	43.60	8.8?	20.3
15	"	62.60	7.90	12.6	67.40	7.40	11.0	71.20	11.00	15.4
16	"	94.10	5.40	5.7	39.10	10.38	26.6	16.40	6.83	41.5
17	Staph. epid.	1.98	0.48	24.3	2.40	1.27	52.7	1.65	0.18	10.9
18	"	57.70	8.67	15.0	[illegible]	6.93	13.4	62.30	9.34	15.0
19	" "	158.50	28.55	18.0	179.60	28.39	1?.8	192.60	2?.45	13.7

RESULTS

Bacterial Counts by the Different Methods

The bacterial counts in urines containing Gram negative or Gram positive bacteria were determined in parallel by the loop method and the pour plate method of *Kass* and of *Merrit* respectively.

Bacterial counts were first performed in ten subsamples from each of 19 urines by each of the three methods. The mean count, the S.D. and the S.D. for the subsamples of each urine were calculated for each method, the results being given in Table 1. The difference between the mean counts obtained by the different methods was tested by the t test. The results are given in Table 2.

TABLE 2

t Test on Significance of Differences between Mean Counts Obtained by Three Different Methods of Urine Culture

Urine no.	Pairs of methods compared		
	Loop/Kass	Loop/Merrit	Kass/Merrit
1	***	N.S.	N.S.
2	***	***	N.S.
3	**	***	N.S.
4	N.S.	*	N.S.
5	***	***	***
6	***	***	N.S.
7	N.S.	***	*
8	N.S.	***	*
9	***	**	**
10	***	**	N.S.
11	***	***	N.S.
12	***	***	N.S.
13	***	N.S.	**
14	***	***	N.S.
15	N.S.	N.S.	N.S.
16	***	***	***
17	N.S.	N.S.	N.S.
18	N.S.	N.S.	**
19	N.S.	*	N.S.

Urine nos. correspond to the same nos. in Table 1. Signs for p values:
N.S. (not significant) $p > 0.05$
* $0.05 > p > 0.01$
** $0.01 > p > 0.001$
*** $p < 0.001$

For the urines containing Gram negative bacteria (urines no. 1–12), the mean count obtained by the loop method is for *most of the urines* significantly lower than that obtained by the two other methods. The *corresponding* mean counts obtained by the two pour plate methods do not differ significantly for most of the urines.

For the urines containing Gram positive bacteria (urines no. 13–19) *the loop method* gives the same mean count as that obtained by the two

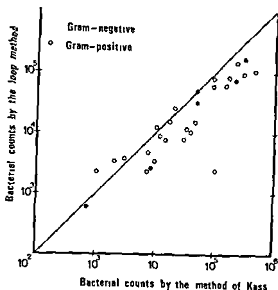


Fig. 1

Bacterial counts of urines performed by the loop method and the method of *Kass*
Bacterial counts by the loop method

other methods or a significantly higher one. It is noteworthy that the latter finding is especially evident in the case of the urines containing Streptococcus faecalis.

The results given in Table 1 also show that the variation in the bacterial counts of each of the urines as expressed by the C. V. is in general lowest for the loop method. This difference is most clearly seen in the urines of low bacterial concentration.

The bacterial counts of 43 urines chosen at random were then determined by the three methods. The counts obtained by the loop method and that of *Kass* were compared as shown in Fig. 1. Each urine contained only a single bacterial strain, 16 of the urines representing Gram negative and 27 Gram positive bacteria. It will be seen from the figure that the loop method tends to give somewhat lower counts than the method of *Kass* when the urines have a high bacterial concentration. For the urines of lower bacterial contents there is good agreement between the results of the two methods. A comparison of the results obtained by the loop method and the method of *Merritt* is omitted here as the results are approximately the same as those referred above.

Some of the factors which may be behind this difference in the counts obtained by means of the three methods are examined below.

The Colony Number Obtained by Pour Plate Technique Compared to that Given by Surface Spreading on Solid Media

Six urines, 3 containing Gram negative and 3 Gram positive bacteria, were each diluted 1 : 100 with broth as described for the method of

kass Ten subsamples of 0 10 ml of each dilution were transferred by pipette to ten plates of solid agar and spread by a loop Ten subsamples were in parallel transferred to ten dishes and poured using melted cooled agar After incubation the bacterial count of each subsample was made

For each urine the mean bacterial count the S D and the C V were calculated for each of the two techniques used The results are presented in Table 3

TABLE 3

Comparison of Bacterial Mean Counts (of Ten Subsamples) of Bacteria in Urines Examined by the Pour Plate Technique and by Surface Streaking on Solid Media Using the Same Agar t test on Differences between Mean Counts

Urine no	Bacteria	Pour plate (agar) I			Solid media (agar) II			t test I/II p value
		Count $\times 10^3$	S D $\times 10^3$	C V %	Count $\times 10^3$	S D $\times 10^3$	C V %	
1	E coli	23 3	6 09	26 3	29 6	3 03	13 4	$0 8 > p > 0 7$
2	E coli	43 8	8 75	20 0	50 9	6 46	12 7	$0 1 > p > 0 05$
3	Klebsiella	58 4	6 8	11 7	62 5	7 31	11 7	$0 3 > p > 0 2$
4	Str faec	25 7	4 19	16 3	38 3	7 50	19 6	$p < 0 001$
5	Str faec	67 4	7 43	11 1	69 9	9 14	13 1	$0 6 > p > 0 5$
6	Staph epid	61 6	6 93	13 4	60 7	7 20	11 9	$0 02 > p > 0 01$

In the case of one urine (Table 3) containing *Streptococcus faecalis* (Str faecalis) and one containing *Staphylococcus epidermidis* (Staph epidermidis) there is a significant difference in the mean counts the surface technique giving the higher count For the four other urines no significant difference is found although a tendency in the same direction can be noted in three of these It is also noteworthy that the two techniques give about the same variation of the subsample counts as that expressed by the C V for all except two of the urines For these two urines which contained *Escherichia coli* (E coli) a somewhat greater variation seems to be related to the pour plate technique

With regard to this comparison of different techniques it should also be mentioned that the colonies grown after surface spreading of urine on solid media were the easiest to detect On the other hand an exact count of these colonies was sometimes more difficult to perform because of a tendency to confluence

This examination seems to show that there is no marked difference between the bacterial counts obtained from the same medium whether the surface spreading or pour plate technique is used It should however be noted that the pour plate technique tends to give slightly lower counts at least in some urines

TABLE 4

Bacterial Mean Counts Obtained by the Loop Method Using Blood Agar and Agar Respectively and Bacterial Mean Counts from the Same Urines by the Pipette Technique Using Agar Surface Spreading Used for all Cultures. t test on the Differences between Mean Counts of Bacteria for the Two Media by the Loop Technique and the Differences between Mean Counts by the Loop Technique and the Pipette Technique Using the Same Agar

Urine no.	Bacteria	Transferred by loop						Transf. by pipette			t test	
		Blood agar (A)			Agar (B)			Agar (C)			A/B	B/C
		Count × 10³	S.D. × 10³	C.V. %	Count × 10³	S.D. × 10³	C.V. %	Count × 10³	S.D. × 10³	C.V. %	p value	p value
1	E. coli	13.3	2.50	18.8	13.2	2.86	21.7	22.6	3.03	13.4	$p > 0.9$	$p < 0.001$
2	E. coli	33.0	4.32	13.1	29.6	3.60	12.2	50.9	6.40	12.7	$0.1 > p > 0.05$	$p < 0.001$
3	Klebsiella	49.0	6.99	14.3	50.3	5.14	10.2	62.5	7.31	11.7	$0.6 > p > 0.5$	$p < 0.001$
4	Str. faec.	34.2	4.05	11.8	26.1	4.10	15.7	38.3	7.50	19.6	$p < 0.001$	$p < 0.001$
5	Str. faec.	62.6	7.92	12.7	14.8	3.26	22.0	69.9	9.10	13.1	$p < 0.001$	$p < 0.001$
6	Staph. epid.	57.9	8.57	14.8	52.8	5.23	9.9	60.7	7.20	11.9	$0.2 > p > 0.1$	$0.02 > p > 0.01$

The Influence of the Composition of Media on Bacterial Growth

A comparison was first made between the growth of bacteria from different urines on two different solid media the agar used in the Kass method and the blood agar used in the loop method Ten subsamples of each urine were transferred to and spread by loop on ten agar plates and ten blood agar plates respectively The data obtained for each urine with each of the two different media are given in Table 4 (Parts A and B)

The results show no significant difference between the two media with regard to the colony numbers which represent the urines containing Gram negative bacteria and the urine containing *Staph epidermis* *Str faecalis* however seems to grow better on blood agar medium which gives significantly higher mean counts than those obtained with agar The C V is about the same for all urines irrespective of the medium used with the exception of one containing *Str faecalis* For this urine a markedly higher C V was related to the growth on agar medium than on blood agar medium

The growth of Gram negative bacteria on blood agar and on lactose agar was then compared As above ten subsamples of each urine were transferred to and spread on each of the two solid media The mean bacterial count the S D and the C V *for each of the 12 urines*, as obtained with blood agar and lactose agar plates are given in Table 5

For 10 of the 12 urines there is no significant difference in the corresponding mean counts obtained with the two media For one urine containing *E coli* the growth on blood agar gives a significantly higher bacterial mean count than the growth on lactose agar and for one urine containing *Klebsiella species* (Klebsiella) the contrary finding is

TABLE 5

Bacterial Mean Counts of Ten Subsamples of Urines Containing Gram Negative Bacteria Spread on Blood Agar and Lactose Agar Plates respectively t test on the Significance of Differences between Mean Counts on the Two Media

Urine no	Bacteria	Blood agar			Lactose agar			t test p value
		Count × 10³	S D × 10³	C V %	Count × 10³	S D × 10³	C V %	
1	E coli	4 41	0 45	10 1	4 05	0 56	13 8	0 2 > p > 0 1
2		13 30	2 50	18 8	9 90	2 10	21 2	0 01 > p > 0 001
3		33 20	4 39	13 2	34 60	4 91	13 9	0 6 > p > 0 5
4	"	35 80	6 99	19 5	38 20	6 58	17 2	0 5 > p > 0 4
5		49 70	9 65	19 4	45 00	7 36	16 4	0 3 > p > 0 2
6	"	62 60	9 42	15 0	62 50	6 70	10 7	p > 0 9
7	Klebsiella	2 00	0 82	41 0	2 20	0 63	28 6	0 6 > p > 0 5
8		9 70	1 42	14 6	9 50	1 18	12 4	p > 0 9
9		10 80	1 40	13 0	9 60	1 51	15 7	0 8 > p > 0 7
10		19 20	4 05	21 1	24 10	3 14	13 0	0 01 > p > 0 001
11		27 40	3 69	13 5	29 30	2 45	8 4	0 2 > p > 0 1
12		49 00	7 21	14 7	45 40	6 93	15 3	0 3 > p > 0 2

made It seems permissible to conclude that Gram negative bacteria in general grow equally well on both media

A comparison was also made of the growth obtained with tryptic digest agar medium and with agar medium Urines were diluted 1 100 with saline (0 9 per cent NaCl) From each urine twenty subsamples each of 0 10 ml were transferred by pipette to twenty dishes ten being poured with agar and ten with tryptic digest agar The data obtained for each urine with the two media are given in Table 6

For two urines containing *Str faecalis* and *Staph epidermis* respectively a significantly higher mean bacterial count was given by tryptic digest agar than by agar alone For the other six urines the two media gave no significant difference with regard to the colony numbers

TABLE 6

Bacterial Mean Counts by the Pour Plate Method Using Tryptic Digest Agar and Agar Respectively t test on Significance of Differences between Mean Counts Obtained with the Two Media

		Pour plate						
		Tryptic digest agar			Agar			
Urine no	Bacteria	Count × 10^3	S D × 10^3	C V %	Count × 10^3	S D × 10^3	C V %	t test p value
1	E coli	21 00	3 80	18 1	23 20	6 10	26 3	0 4 > p > 0 3
2	E coli	42 90	5 84	13 6	43 80	8 75	20 0	0 8 > p > 0 7
3	Klebsiella	63 20	7 36	11 6	58 40	6 85	11 7	0 2 > p > 0 1
4	Str faec	71 20	11 00	15 4	67 40	7 40	11 0	0 4 > p > 0 3
5	Str faec	31 10	3 93	12 6	[illegible]	4 19	16 3	0 01 > p > 0 001
6	Staph epid	62 30	9 34	15 0	51 60	6 93	13 4	0 01 > p > 0 001

The Transfer of Urine by Loop Compared to the Transfer by Pipette

Ten subsamples of each of 6 urines were transferred by the 0 001 ml loop and ten subsamples of 0 10 ml of the same urines diluted 1 100 with saline were transferred by pipette to agar plates and spread by loop

The results obtained for each series of subsamples are presented in Table 4 (Parts B and C)

A comparison of the two mean counts for each urine shows that the pipette technique gives a considerably higher mean count for all urines than the loop technique

This examination shows that there is without doubt a difference between the number of bacteria transferred by the two techniques

The question of what lies behind this difference will be discussed later

TABLE 7

Bacterial Mean Counts of Ten Urine Specimens Mixed by Conventional Shaking by Hand and by Motor Shaker for 1 and 5 Minutes Respectively t test on Significance of Differences between Mean Counts of Specimens Mixed by the Three Shaking Methods

Urine no	Bacteria	Conventional shaking I			Motor shaker 1 min II			Motor shaker 5 min III			t test	
		Count × 10^3	S D × 10^3	C V %	Count × 10^3	S D × 10^3	C V %	Count × 10^3	S D × 10^3	C V %	I/II p value	II/III p value
1	E coli	49 38	9 48	19 2	47 92	4 30	9 1	41 21	7 72	18 7	$0 6 > p > 0 5$	$0 05 > p > 0 02$
2	Klebsiella	10 [illegible]	1 35	12 6	10 98	0 92	8 4	9 67	2 23	23 0	$0 6 > p > 0 5$	$0 3 > p > 0 2$
3	Str faec	93 65	5 28	5 6	94 96	12 89	13 6	97 78	8 51	8 7	$0 8 > p > 0 7$	$0 3 > p > 0 2$
4	Staph epid	1 98	0 48	24 3	1 77	0 49	27 6	1 84	0 41	22 1	$0 4 > p > 0 3$	$0 5 > p > 0 4$

The Distribution of Bacteria in Urine after Various Methods of Shaking

From each of 4 urines ten subsamples were taken by the loop after conventional shaking of the test tubes by hand and ten subsamples after shaking the tubes by a motor shaker for 1 and 5 minutes respectively. Each subsample was spread by the loop on blood agar plates. The data for each series of subsamples are presented in Table 7.

The results show no significant difference in the mean counts for the three series compared with the exception of urine no. 1, where the mean counts after conventional shaking and 5 minutes shaking differ ($t = 2.111$, $f = 18$, $0.05 > p > 0.02$). The results do not indicate any systematic variation of the mean counts related to the shaking procedure. A more uniform distribution of the bacteria in the urine samples is thus not obtained by use of a motor shaker than by the conventional shaking of the tubes by hand. This result indicates that after conventional shaking the distribution of bacteria is the same irrespective of the level in the tubes from which the subsamples are taken.

DISCUSSION

Theoretically, the best method of quantitative urine culture is that giving the best reproducibility of the counts and the highest counts for all strains of bacteria. For a clinical purpose the method should also be convenient and not too time consuming or expensive. Of the three methods compared in this work the loop method is undoubtedly the most convenient for clinical and epidemiological work.

The reproducibility as expressed by the S.D. and the C.V. of a series of ten subsamples examined in parallel by the three methods seems to be somewhat lower for the two pour plate methods than for the loop method when the urine contains few bacteria. For urines with a bacterial count near the commonly accepted limit between contamination and real infection the reproducibility seems to be about the same for all three methods irrespective of whether the bacteria are Gram negative or Gram positive. However, it must be mentioned that the variation due to the dilution procedure in the pour plate methods is not included in the reproducibility results presented above. This variation is supposed not to be negligible (2, 10). It is therefore reasonable to believe that the loop method gives the same or even better reproducibility of the bacterial counts. For all three methods, however, the variation found is of such an extent that it must be taken into account when borderline counts are obtained.

Furthermore, the best method is supposed to be that giving the highest bacterial counts and thus reflecting most correctly the number of viable bacteria in the urine. The present investigation does not give an answer of general validity for all urines containing bacteria. For urines containing Gram negative bacteria the pour plate methods gave

significantly higher counts than the loop method. This fact seems to be valid irrespective of the number of bacteria in the urine but the relationship between corresponding counts obtained by the pour plate methods and by the loop method is not constant. When corresponding mean counts obtained by the two pour plate methods are compared they are found to diverge relatively little although a more significant difference is found in the case of some urines. Thus neither of these two methods seems to have an advantage over the other with regard to the count of gram negative bacteria. When the corresponding bacterial mean counts obtained by the three methods are compared for urines containing Gram positive bacteria the loop method seems to give the same or even higher counts than the other methods. The higher counts are found especially in the case of urines containing *Str faecalis*.

Some of the factors which may contribute to the above described difference between the bacterial counts obtained by the three methods were examined. The composition of the media no doubt influences the bacterial counts (Table 4 and 6) when the urines contain Gram positive bacteria. The blood agar medium used in the loop method gives a significantly better growth of these bacteria than the other media especially favouring strains of *Str faecalis*. The tryptic digest agar used by *Merrit* seems to give somewhat better growth conditions for Gram positive bacteria than ordinary agar. With regard to the Gram negative bacteria all media gave approximately the same growth as expressed by the colony numbers. The different media used in the three methods may thus explain some of the differences between the counts of Gram positive bacteria but not the relatively larger differences observed between the counts of Gram negative bacteria obtained by the pour plate methods and by the loop method.

The reason for the difference between the counts was also sought in the different culture techniques—spreading on solid media and pour plate. For some urines there seems to be a tendency to lower counts when the pour plate technique is used and for two urines containing *Str faecalis* and *Staph epidermidis*, respectively the difference was significant. For the other urines however including those containing Gram negative bacteria the same bacterial count was obtained by the different techniques. This finding is in accordance with those of *Dalton* (3) and *Hinkel* (6) and does not at all explain the different counts of Gram negative bacteria obtained by the loop method and by the pour plate methods. Neither can this difference be explained by an uneven distribution of the bacteria in the urine samples (Table 7).

Most probably the explanation of the considerable difference between counts made by the loop method and by the pour plate method is that a smaller number of bacteria is transferred to the media by the loop than by the pipette. The standardization of the loops was performed with physiological urine (4) and the possibility cannot be excluded that smaller and more variable volumes of urine containing bacteria

are transferred. If this is the case the reason is probably not related to the specific weight (4) or viscosity (5) of urines containing bacteria differing from physiological urine. Other as yet unknown factors may possibly contribute to a reduction or variation in the volumes of different urines transferred by loops.

It seems, however, more probable that the fluid volumes transferred to the medium by loops are correct but that the number of bacteria transferred is smaller than that which would correspond to the fluid volume. One may well imagine that due to its physical and chemical properties, the cell surface (14) will have a different electrokinetic reaction—repulsive or attractive—with the metal of the loop than with the glass of the pipette. The adhesiveness of piliated Gram negative bacteria commonly found in infected human urinary tracts (1) may also play a role. Further research is necessary to evaluate the influence of these factors.

As a general conclusion it may be said that bacterial counts in urines depend to some extent upon the method used. A careful comparison of counts obtained by different methods should therefore be made. None of the three methods here compared seems to be the method of choice for all purposes. The pour plate methods reflect most correctly the number of Gram negative bacteria in the urine, but the loop method seems to give a better reproducibility of counts. In addition the loop method has the advantage of being more convenient and less time consuming than the pour plate methods. As long as no ideal method exists, the loop method can be said to be a convenient method for clinical and epidemiological work.

SUMMARY

A comparison was made between the bacterial counts in urines obtained by three different methods: the loop method and the pour plate method of *Kass* and of *Merrit* respectively. Significantly lower counts of Gram negative bacteria were obtained by the loop method than by the pourplate methods. Urines containing Gram positive bacteria, however, gave identical or even higher counts by the loop method than by the others. The reproducibility in repeated counts seemed to be best for the loop method.

None of the methods seemed ideal for the performance of bacterial counts in all urines. The loop technique, however, was the most convenient and least time consuming and thus the most useful for clinical and epidemiological work.

REFERENCES

1. *Brinton, C. C. Jr.:* Contributions of pili to the specificity of the bacterial surface and a unitary hypothesis of conjugal infectious heredity. In: *The specificity of cell surfaces.* Edited by Davis, B. D. & Warren, L. Prentice Hall Inc., Englewood Cliffs, N.J. 1967, pp. 37–70.

2 *Cattell W R & Jefford M J* Bacterial examination of urine Brit Med. J *1* 97–100 1963
3 *Dalton H P* The use of spread plates in urine colony counts Am J Med techn *29* 247–248 1963
4 *Haugen J Strom O & Ostervold B* Bacterial counts of urine I Acta path. microbiol scand *74* 391–396 1968
5 *Haugen J Ström O & Ostervold B* Unpublished data
6 *Hinkle N M Partin J C & West C D* Diagnosis of acute and chronic pyelo nephritis in children Am J Dis Child *100* 333–340 1960
7 *Hoeprich P D* Culture of the urine J Lab & Clin Med *56* 899–907 1960
8 *Kass E H* Personal communication
9 *Lystad A & Gardborg O* Kvantitative urinanalyser i den bakteriologiske laboratoriediagnostikk Tidskr D norske lægefor *82* 57–60 1962
10 *McGeachie J & Kennedy A C* Simplified quantitative methods for bacteriuria and pyuria J clin Path *16* 3 –38 1963
11 *Merritt A D & Sandford J P* Sterile voided urine culture J Lab & Clin Med *56* 899–907 1960
12 *Revel J P & Ito S* The surface components of cells Pp 211–234 in The specificity of cell surfaces Edited by Davis B D & Warren L. Prentice Hall Inc Englewood Cliffs N J 1967

Acta path microbiol scand 77 162-166 1969

Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt Oslo Norway
the Department of Clinical Chemistry Danderyds Sjukhus Danderyd Sweden and
the Institute of Pathological Anatomy Rikshospitalet Oslo Norway

BILE ACID TRANSFORMING MICRO ORGANISMS IN RATS WITH AN INTESTINAL BLIND SEGMENT

By

TORE MIDTVEDT ARNE NORMAN and KNUT NYGAARD

Received 24 III 69

The presence of a blind loop or a blind segment on the small intestine either spontaneously occurring or experimentally produced may be associated with impaired fat absorption (for review see 11) A pathogenic role of bacteria in this condition is strongly suggested by various observations Thus bacterial overgrowth occurs in the small intestine (2, 11) increased amounts of free bile acids is present in the small intestine (15) the steatorrhea usually responds to antibacterial therapy (1 4 13) and germfree blind loop rats kept on a standard diet excrete 4–8 times less fatty acids per gram of food intake compared with conventional blind loop rats (5)

However in spite of these indications of a microbial interaction in the blind loop syndrome little is known about the more specific alterations of the intestinal flora in this condition The aim of the present investigation was to estimate the frequency of aerobic anaerobic and *bile acid transforming* micro organisms in the intestinal contents of rats with a blind segment on the jejunum as compared with control rats The transformation processes studied were splitting of glycine and taurine conjugates elimination of the hydroxyl group at C 7 and oxidation of the hydroxyl groups at C 3 and C 7 to keto groups

MATERIALS AND METHODS

Animals and Diet

Adult male rats (300–400 g) fed on a commercial rat food (12) and water *ad libitum* were used Three animals were operated and three unoperated rats [illegible] as controls

Surgical Procedures

Laparotomy was performed *under ether alcohol narcosis* The mid portion of the jejunum was transected and the proximal end closed Continuity was re established

---

This investigation was supported by grants from *Norsk Medisinalfond* [illegible] *Swedish Medical Research Council* (602) and *Stockholms Läns Landsting*

with an end to side anastomosis between the intestine 8 cm proximal to the end closed and the end of the distal portion thereby creating an 8 cm long self filling blind segment

Method of Sampling

Sampling was performed 5–6 weeks after the operation The abdomen was opened under narcosis the intestine divided aseptically and the intestinal contents extruded into sterile tubes In the operated rats samples were taken at the following sites a the blind segment b the ileum and c the caecum In the control rats samples were taken at corresponding site

Bacteriological Procedures

The techniques used for aerobic and anaerobic cultivations and bile acid transformations were as described elsewhere (9)

Chemical Procedures

As regards sources and references to methods for synthesizing 24 C^{14} labelled and unlabelled free and conjugated bile acids see (7) as to the description of the techniques employed in isolation and determination of the metabolites formed as well as the names and abbreviations of these metabolites see (10)

RESULTS

Bacterial Counts in Intestinal Contents

Table 1 shows the number of anaerobic and aerobic micro organisms present in contents taken from the blind segment ileum and caecum in the operated rats (rats no B1–B3) and in the contents from jejunum ileum and caecum in the control rats (rats no C4 C6)

In the operated rats the total number of microbes was of the same order of magnitude (10^9–10^{11}) in the blind segment ileum and caecum contents The values obtained under aerobic culture conditions were at least one dilution step lower than those obtained anaerobically in all samples except one

In the control rats the number of anaerobic micro organisms was 10–10^5 in jejunum contents and increased to 10^5–10^9 and 10^9–10^{10} in the ileum and caecum contents respectively The values obtained aerobically were here also at least one dilution step lower in all cases except one

Splitting of Conjugates

The number of micro organisms capable of splitting conjugated bile acids was equivalent to the number of anaerobes in all samples except one In that case deconjugating microbes were present in the next highest dilution step showing anaerobic growth

Oxidation of Hydroxyl Groups at C 3 and C 7

The number of organisms capable of oxidizing the hydroxyl group at C 7 to a keto group was of the same order of magnitude in the blind segment ileum and caecum contents from the operated rats (10^9–10^{11})

The control rats showed low values of such organisms in jejunum contents (10^4–10) and higher values in ileum and caecum contents (10^5–10^9)

TABLE 1

Microbial Growth and Bile Acid Transformation in Dilution of Rat Intestinal Contents taken from Jejunum Ileum and Caecum in Blind Loop and Control Rats

No of microorganisms per gram intestinal contents

Rat no	Sample from	Anaerobes	Aerobes	Microorganisms capable of: Splitting Glycocholic acid	Splitting Taurocholic acid	7 keto formation	3 keto formation	7α dehydroxylation
B1	Blind segment	10^{11}	10^{10}	10^{11}	10^{11}	10^{11}	10^9	10^4
B2		10^{10}	10^9	10^{10}	10^{10}	10^{10}	10^3	10^6
B3		10^{10}	10^9	10^{10}	10^{10}	10^9	10^4	10^4
C4	Jejunum	10^7	10^3	10^7	10	10^5	0	10^2
C5		10^9	10^9	10^9	10^9	10^7	0	0
C6		10^5	10^6	10^5	10^5	10^4	0	0
B1	Ileum	10^{11}	10^{10}	10^{11}	10^{11}	10^{11}	10^7	10^9
B2		10^9	10^8	10^3	10^9	10^9	10^5	10^7
B3		10^{10}	10	10^{10}	10^{10}	10^9	10^3	10^4
C4		10^8	10^6	10^4	10^8	10^8	10^4	10
C5		10^9	10^4	10	10^9	10^9	10	10^2
C6	"	10^5	10^1	10^8	10^4	10^5	0	0
B1	Caecum	10^{10}	10^{10}	10^{10}	10^{10}	10^{10}	10	10^5
B2		10^{11}	10^7	10^{10}	10^{10}	10^{10}	10	10^2
B3		10^{10}	10^8	10^{10}	10^{10}	10	10^6	10^5
C4		10^{10}	10^7	10^{10}	10^{10}	10^8	10^5	10^5
C5		10^9	10^5	10^9	10^9	10^8	10^7	10^4
C6		10^9	10^7	10^9	10^9	10^5	10^3	10^4

B1–B3 = rats with an intestinal blind segment C4 C6 = control rats

Micro organisms capable of oxidizing the hydroxyl group at C 3 were always present although in varying numbers in the blind segment ileum and caecum contents from the operated rats Such organisms were not demonstrated in the jejunum contents from control rats and in the ileum contents from one control rat but were present in the ileum contents from two control rats and in the caecum contents from all the control rats

Removal of the 7α hydroxyl Group

Micro organisms capable of 7α dehydroxylation were always present and in approximately the same numbers in the blind segment ileum and caecum contents from the operated rats (10^4 10^4 10^4 10 and 10^2 10^5) In the control rats 7α-dehydroxylating organisms were absent or present in small numbers only in the jejunum and ileum contents but were always present in the caecum contents

DISCUSSION

The physiological effects of bile acids are mainly exerted in the small intestine and the major part of the microbial transformations starts when the bile acids reach the caecum. It is likely that any alteration in the flora in the small intestine might influence the state of the bile acids and thus also their interactions in intestinal functions. Some of the dysfunctions found in the blind loop syndrome might be due either to a toxic effect upon the intestinal mucosa of bile acid derivatives formed by microbial enzymes or to a reduction of the concentration of conjugated bile acids below that necessary for normal fat absorption.

The toxic derivative in focus has been deoxycholic acid (i.e. the 7α dehydroxylated derivative of cholic acid). Deoxycholic acid has been shown to inhibit the uptake and esterification of fatty acids by the intestinal mucosa (3, 4). However, long term feeding of intact animals with deoxycholic acid does not produce any major alteration in absorption of fatty acids or any histologically demonstrable damage to the mucosa (14). It is, however, well known that 7α dehydroxylated bile acids in normal rats are easily bound to intestinal contents and thus might be inhibited in exerting their toxic effects.

The alternative suggestion that a reduction in the concentration of conjugated bile acids causes impaired fat absorption has been investigated by *Kim et al.* (8). They demonstrated in dogs with experimentally produced blind loop that bacterial deconjugation of bile acids was associated with a marked reduction of normal micelle formation and that a dietary addition of taurocholic acid tended to normalize the fat absorption. Unfortunately, the formation of 7α dehydroxylated bile acids in the blind loop dogs was not investigated.

Comparing the results obtained in the operated rats in this investigation with those found in the control rats, it is evident that the microbial flora in the blind segment and ileum contents in the operated rats has been "caecalized", i.e. as found in caecum contents, both with regard to the numbers of aerobic and anaerobic organisms present as well as to the capacity of deconjugating and 7α dehydroxylating bile acids. It is reasonable to believe that these reactions are performed in the small intestine by the organisms present. The results do not, however, provide any information about the extent to which the two reactions take place in the small intestine of the operated rats or about the role they play in the development of the blind loop syndrome.

SUMMARY

The number of bile acid transforming intestinal micro organisms was determined in rats with a blind jejunal segment. There was a distinct increase of organisms capable of splitting glycine and taurine conjugates, eliminating the hydroxyl group at C 7 and oxidizing the hydroxyl

groups at C 3 and C 7 to keto groups in the blind segment and the ileum as compared to findings in unoperated rats

REFERENCES

1 *Badenoch J* Steatorrhea in the adult Brit med J 2 963–974 1960
2 *Bishop R* Bacterial flora of the small intestine of dogs and rats with intestinal blind loop Brit J Exp Path 44 189–196 1963
3 *Dawson A M* & *Isselbacher K J* Studies on lipid metabolism in the small intestine with observations on the role of bile salts J Clin Invest 39 730–740 1960
4 *Donaldson R M* Studies on the pathogenesis of steatorrhea in the blind loop syndrome J Clin Invest 44 1815–1825 1965
5 *Evrard E* *Sacquet E* *Raibaud P* *Charlier H* *Dickinson A* *Eyssen H* & *Hoet P P* Studies on conventional and gnotobiotic rats effect of intestinal bacteria on fecal lipids and fecal sterols Ernährungsforschung 10 257–263 1965
6 *Fry R J M* & *Staffeldt E* Effect of a diet containing sodium deoxycholate on the intestinal mucosa of the mouse Nature 203 1396–1398 1964
7 *Gustafsson B E* *Midtvedt T* & *Norman A* Isolated fecal microorganisms capable of 7α dehydroxylating bile acids J Exp Med 123 413–432 1966
8 *Kim Y S* *Spritz N* *Blum M* *Terz J* & *Sherlock P* The role of altered bile acid metabolism in the steatorrhea of experimental blind loop J Clin Invest 45 956–962 1966
9 *Midtvedt T* & *Norman A* Anaerobic bile acid transforming microorganisms in rat intestinal content Acta path microbiol scand 72 337–344 1968
10 *Midtvedt T* & *Norman A* Parameters in 7α dehydroxylation of bile acids by anaerobic lactobacilli Acta path microbiol scand 72 313–329 1968
11 *Nygaard K* Changes in the intestinal flora after resections and bypass operations on the small intestine in rats Acta chir scand 133 569–583 1967
12 *Nygaard K* Nutrition and absorption after resection and bypass operations on the small intestine in rats Acta chir scand 134 63–77 1968
13 *Panish J F* Experimental blind loop steatorrhea Gastroenterology 45 394 399 1963
14 *Tabaqchali S* & *Booth C C* Jejunal bacteriology and bile salt metabolism in patients with intestinal malabsorption Lancet (II) 12 15 1966
15 *Terz J J* *Kim Y S* *Spritz N* *Blum M* & *Sherlock P* Experimental production of blind loop steatorrhea and a study of its pathogenesis Surgical Forum 16 349–350 1965

Acta path microbiol scand 77 167–168 1969

BRIEF REPORTS

THE EFFECT OF ANTILYMPHOCYTE SERUM ON VIRAEMIA AND SERUM INTERFERON OF MICE INFECTED WITH WEST NILE VIRUS

B *Sten Haahr*

Heterologous antilymphocyte serum (A L S) has been used in an increasing degree during the past few years as an effective suppressant of cellular immunity (7)

Suggestive evidence of increased susceptibility to viral infection has been obtained in connection with A L S therapy and such infections could be a problem with prolonged treatment with A L S (1)

This increased susceptibility to viral infections may be based on suppression of cellular immunity (5) One investigation has shown that interferon production is not affected (6) but in general, the possible influence of A L S on interferon production has apparently not been studied As lymphocytes have previously been found to be good interferon producers (9) it was considered of value to further study interferon induction in connection with viral infections in mice given A L S

The experimental system used was essentially the same as described in previous papers (2 3) Male and female mice of a non inbred strain weighing 20–22 g were used in these experiments The virus content of the serum was determined by intracerebral inoculation into mice aged 2 to 4 days of 10 fold serial dilutions in PBS Virus titre was expressed as the exponent of the logarithmic (log $_{10}$) dilution per 20 microl which caused death in 50 per cent of the animals as calculated by the method of *Karber* (8) Determination of antiviral activity was performed by the plaque inhibition method in secondary cultures of mouse embryonic cells using Semliki Forest Disease virus as challenge virus Interferon titres expressed as units per 200 microl of serum were recorded as the reciprocal of the highest dilution which reduced by 50 per cent the number of plaques counted in the controls i e 50 per cent plaque depressing dose (PDD$_{50}$/200 microl)

A L S was produced in rabbits as follows Cells used for immunization were prepared from axillary and inguinal lymphnodes from adult male mice The tissue was minced with knives and subsequently treated with a 0 01 per cent trypsin solution (*Trypure*®) for four or five periods of 10 minutes The first suspension of cells was discharged because of an ample content of erythrocytes Subsequently a suspension consisting almost entirely of lymphocytes was obtained After centrifugation at 2000 rpm for 10 min the trypsin solution was decanted and the cells resuspended in PBS for a final concentration of approximately 2 × 10^8 cells/ml One ml of this suspension was mixed with one ml of complete Freund's adjuvant and injected subcutaneously into rabbits at five to eight different sites for four successive days Four weeks later 2 × 10^8 lymphocytes were administered intravenously and one week later the rabbits were bled Normal serum (N R S) was obtained from non treated rabbits Serum was not inactivated and was stored at −20 C Mice were given 0 5 ml A L S or N R S intraperitoneally 1 to 2 hours before virus inoculation In experiments lasting several days serum was given daily and always 1 to 2 hours before exsanguination of the mice under study Leucocyte and differential counts were done daily 1 to 2 hours after A L S or N R S administration Already after the first A L S injection and continuing after the subsequent injection the lymphocyte count in mice given A L S was approximately 1/4 to 1/5 of the lymphocyte count in mice given N R S, whereas there was no change in the number of polymorphonuclear leucocytes

In the main experiment two different doses of West Nile virus as determined by intracerebral inoculation in weanling mice were used In several experiments virus in a dose of 10^6 LD$_{50}$ was injected intraperitoneally The serum was then studied

Received 21 vi 69 from the Institute of Medical Microbiology University of Aarhus 8000 Aarhus C Denmark

for interferon activity and virus content 18 hours after inoculation—about the time of maximum circulating interferon following intraperitoneally inoculation of a large virus dose (4) In Table 1 a a typical experiment is presented A rather appreciable reduction in circulating interferon was found together with slightly increased virus titre in the mice given A I S

TABLE 1 a

Virus and Interferon in Pooled Serum from 8 Mice Given A I S and N R S and Inoculated with 10 and 10³ LD₅₀ West Nile Virus Respectively

Hours after inoculation of 10 LD_{50}	A I S		N R S	
	Virus	Interferon§	Virus	Interferon
18	2 15	8	1 85	48

TABLE 1 b

Hours after inoculations of 10^3 LD_{50}	A L S		N R S	
	Virus	Interferon	Virus	Interferon
24	<1	<4	1 5	<4
48	<1	<4	2 7	
72	2 5	<4	2 9	4
96	3 3	≦4	3 3	32
120	<1	<4	<1	4

Virus log LD_{50}/20 microl
§ Interferon PDD_{50}/200 microl

In other experiments where 10^3 LD_{50} was given the viraemia and the amount of circulating interferon was studied during continuous A L S or N R S administration In Table 1 b which gives the results of a typical experiment the amount of circulating interferon remained reduced in the mice given A I S in comparison with the mice given N R S *Concomitantly a reduction in viraemia was found during* the first two days together with a smaller reduction later in the mice given A L S

These studies thus showed a reduction in circulating interferon in mice given A I S and inoculated with West Nile virus

The results obtained seem to suggest that circulating interferon in mice inoculated with the arbovirus used here is to a large degree produced by circulating lymphocytes and/or lymphoid tissue Further study of this question including study of other viruses and animals is in progress at our laboratories

The increased viraemia after the inoculation of a large amount of virus and the reduced viraemia after inoculation of a smaller amount of virus in mice given A L S was seen in all the experiments performed The explanation of this can at present only be a speculation These findings make it difficult to say anything definite about the effects of A I S administration on the course of viral infections This question is also under study

References 1 *Abaza H M Nolan B Watt J C & Woodruff M F A* *Transplantation* 4 618–632 1966 —2 *Haahr S* Acta path microbiol scand 74 445 457 1968 —3 *Haahr S* Acta path microbiol scand 73 304 319 1968 4 *Haahr S* unpublished results —5 *Hirsch M S & Murphy F A* Lancet ii 3 40 1968 —6 *Hirsch M S Nahmias A J Murphy F A & Kramer J H* J exp Med 128 121 130 1968 —7 *James K* *Clin exp Immunol* 2 615 631 1967 —8 Kahl r (Arch G Exper Path u Pharmakol 160 480–490 1931 —9 *Wheelo k E F* J Bact 92 1415–1421 1966

Acta path microbiol scand 77 169-171 1969

INCREASE IN SENSITIVITY OF THE RHODAMINE B METHOD FOR KERATINIZATION BY THE USE OF FLUORESCENT LIGHT

By *Finn P Clausen* and *Erik Dabelsteen*

Rhodamine B was first used as a stain for cornification by *Martinotti* 1924 who presented two methods for eleidine and keratogene substances in which the dye was used together with either monophenylrosanilin or Victoria blue

In 1968 *Lisberg* published a method for demonstrating cornified tissue which was claimed to be extremely specific Rhodamine B was used at pH 3 6 after blocking of basophilic substances by toluidine blue

The present authors tried *Lisberg's* method on formalin fixed paraffin embedded sections of oral mucosa and keratocysts but found that they stained rather faintly and not absolutely specifically As rhodamine B has been used as a fluorochrome for many years (*Harms* 1965) it was decided to try to enhance the sensitivity of the method by means of fluorescence microscopy

Formalin fixed paraffin embedded sections of biopsies from oral leucoplakias keratocysts oral lichen planus and oral carcinomas containing keratinized layers as evidenced in haemalum eosin stainings were used as test objects In addition scrapings from the palatal and buccal mucosa were stained after fixation in 96 per cent ethyl alcohol

The rhodamine B was Gurr's Michrome no 407 and the toluidine blue was Toluidinblau nach Hoyer" Sections were mounted in Eukitt Fluorescence microscopical examination was made with a Leitz Orthoplan microscope fitted with an Osram HBO 200 W lamp A 5 mm BG 12 filter was used for excitation and a Leitz K 530 filter as a barrier

In all sections examined the sensitivity of the rhodamine B method was significantly enhanced by fluorescent blue light The keratinized layers showed an intense brilliant yellow secondary fluorescence (Fig 1 A + B) A distinct fluorescence was seen even in areas where the keratinized layer was very thin and the staining very difficult to evaluate in white light (Fig 2 A + B) Unstained sites showed a greenish fluorescence a sharp contrast between keratinized and unkeratinized sites therefore being readily obtained Unfortunately striated muscle tissue collagen fibres and erythrocytes are often partly stained by rhodamine B the reaction being seen clearly by white light These limitations of the method do not however influence the interpretations of the keratinization of the epithelium

Leucoplakias with orthokeratinized epithelium showed an intense rhodamine B positive reaction confined to the keratinized layers with a distinct border adjacent to the granular layer The reaction was more sharply demarcated and more uniform in fluorescent light than in white light Some of the prickle cells showed a weak cytoplasmic reaction in fluorescent light which was hardly recognizable in white light

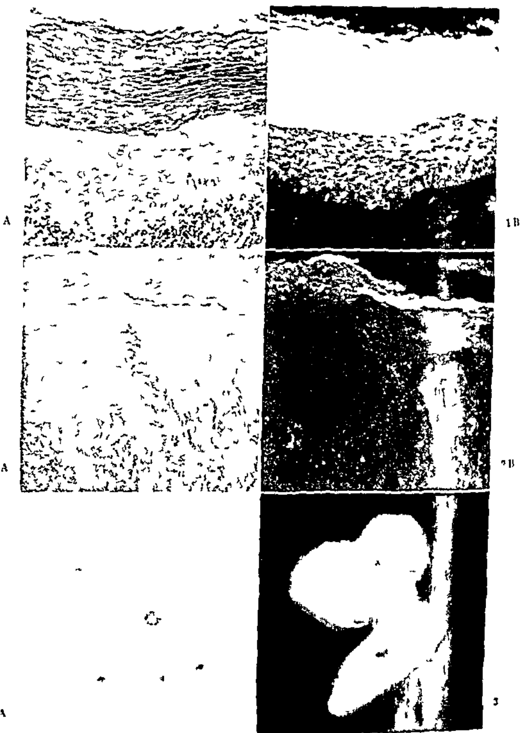
Leucoplakias having a parakeratinized epithelium and a stratum granulosum exhibited in visible light an irregularly rhodamine B positive keratinized layer with negative pyknotic nuclei and in fluorescent light a regularly positive sharply demarcated keratinized layer with negative nuclei

Incomplete parakeratosis (*Weinmann & Meyer* 1959) could be seen in some leucoplakias These showed a weak rhodamine B staining of the parakeratinized parts

---

Received 7 xii 69 *from the Department of Oral Pathology Royal Dental College DK 2100 Copenhagen Ø Denmark*

Supported in part by a grant DE 1358 from the *National Institute of Dental Research National Institutes of Health U S P H S Bethesda Maryland*


A
1B
A
2B
A
3

of the surface layer in visible light whereas the reaction was intense in fluorescent light (Fig 2 A + B)

Sections of lichen planus showed the same reactions in similar sites with regard to type and degree of keratinization Carcinomas showed a rhodamine B positive reaction within ortho and parakeratinized keratin pearls With fluorescent light *it was possible to see a varying intensity in different areas of the keratin pearls*

The intensity of the rhodamine B reaction in exfoliated cells of the oral mucosa was so weak in visible light that the method was valueless In fluorescent light however the cells possessed *a comprehensive range of reactions* (*Fig 3 A* + B) Whether the intensity of fluorescence reflects the degree of keratinization and is in closer agreement with soft ray microradiograms than the Papanicolaou staining reaction (*Dabelsteen & Clausen* 1969) remains to be studied

It is not yet known to which substances rhodamine B is attached in the tissues but as *Boerner* (1952) has shown that rhodamine B can be used as a fluorochrome for the demonstration of lipoid (see also *Lillie* 1964 p 115) it is likely that some of the lipoid substances demonstrated in keratinized tissues by *Frithiof & Wersall* (1965) may be involved

References 1 *Boerner Pat ell D* Protoplasma *41* 168–177 1952 —2 *Dabelsteen E & Clausen F P* To be published—3 *Frithiof L & Wersall J* J Ultrastruct Res *12* 371–379 1965 —4 *Harms H* Handbuch der Farbstoffe fur die Mikroskopie Staufen Verlag Paul Bercker Kamp Lintfort 1965 —5 *Lisberg M F* Acta anat 69 52–57 1968 —6 *Lillie R D* Histopathologic technic and practical histochemistry Blakiston Co New York, 1964 —7 *Martinotti L* Z wiss Mikr *41* 209–258 1924 — 8 *Weinmann J P & Meyer J* J invest Derm *32* 87–94 1959

Figs 1–3

Fig 1 A + B Rhodamine B stained section of a leucoplakia from the buccal mucosa of a 59 year old man The epithelium shows a hyperorthokeratinization A is photographed *in visible white light and shows staining* of the keratinized layer The staining is much more intense however in B which is photographed in fluorescent light × 80

Fig 2 A + B Rhodamine B *stained section of a leucoplakia from the buccal mucosa* of a 58 year old man The epithelium shows an incomplete hyperparakeratinization A is photographed in visible white light and B in fluorescent *light The* parakeratinized part of the surface is seen much more distinctly *in the latter × 80*

Fig 3 A + B Rhodamine B stained smear from the buccal mucosa of a 27 year old man with *leucoedema A is* photographed in visible white light and B in fluorescent light In the latter a much more comprehensive range of intensity is seen × 370

Acta path microbiol scand 77 173-175 1969

ABNORMALITIES OF THE COSTOCHONDRAL JUNCTION IN CASES OF PERINATAL DEATH WITH SPECIAL REFERENCE TO HYALINE MEMBRANE DISEASE

By *Bengt Robertson* and *Björn Ivemark*

In a recent histological study of the ribs and lungs in a small series of neonatal autopsy subjects the common association of trabecular rarefaction in the costochondral junction (CCJ) with pulmonary hyaline membranes was recognized (*Robertson* 1969) This finding suggested that some specific type of foetal disorder reflected in the CCJ might be causally related to the development of the idiopathic respiratory distress syndrome This hypothesis is now currently tested in our laboratory along various lines

Material and Methods

The material which was obtained from three hospitals consisted of 50 consecutive neonatal autopsy subjects age range stillborn—two weeks The CCJ was examined in decalcified longitudinal histological sections from the ribs stained with haematoxylin and eosin The lesions in the CCJ were classified according to the following principles (modified from *Cohen* 1948 and *Emery & Kalpaktsoglou* 1967)

Normal The borderline between the cartilage and the metaphysis is straight In the metaphysis there is a regular pattern of longitudinally arranged trabeculae undergoing mineralization The bone marrow spaces are cellular and the capillaries *are in close contact with the end of the well defined pillars of cartilage cells*

Abnormal Two main patterns of growth disturbance were recognized

I The borderline between the cartilage and the metaphysis is irregular The cartilage cells do not form the usual pillars but seem to migrate into the metaphyseal area surrounded by abundant cartilage matrix which prevents the close contact between the cartilage cells and the bone marrow capillaries (matrix banding *Emery & Kalpaktsoglou* 1967) In the metaphysis there is an irregular pattern of fairly broad trabeculae The term chondral dysplasia (CD) was applied to this type of lesion in the CCJ

II The borderline between the cartilage and the metaphysis is straight without matrix banding In the metaphysis there is rarefaction of the trabecular pattern In places the delicate trabeculae bridge multiple columns of cartilage cells (bridging *Emery & Kalpaktsoglou* 1967) Increased osteoclastic activity is observed in the metaphysis which has a porous appearance *The bone marrow spaces appear* hypovascular with reduced haematopoietic activity The term trabecular rarefaction (TR) was applied to this type of lesion in the CCJ

The majority of the ribs could be classified in one of the abovementioned groups but some showed a less distinct or a mixed type of lesion classified as *intermediate* (IM)

Grading The severity of the lesions was graded as slight (+) moderate (++) or marked (+++)

Reactive zone In many ribs especially those with lesions of the TR or IM types the abnormal metaphyseal zone was more or *less demarcated This was particularly* evident in low power fields and the size of the abnormal area arbitrarily referred to as the reactive zone could be measured along *the longitudinal axis* of the rib

Classification grading and measuring of the CCJ lesions were performed inde

---

Received 8 viii 69 *from the Department of Pediatric Pathology Karolinska sjukhuset Stockholm Sweden*

Requests for reprints should be addressed to Bengt Robertson M D Department of Pediatric Pathology Karolinska sjukhuset S 104 01 Stockholm Sweden

TABLE 1

pes and Grades of Lesions in the Costochondral Junction in Relation to Clinical Diagnosis and Autopsy Findings

Diagnosis	Costochondral junction Number of cases										Total
	Normal	Chondral dysplasia			Intermediate			Trabecular rarefaction			
		+	++	+++	+	++	+++	+	++	+++	
lmonary hyaline membranes Idiopathic espiratory distress	1				3	2	1	1	1	3	12
lformations nel pulmonary hypoplasia Pneumonia	1	4	2	1		1			2		11
lformations without pulmonary hypoplasia	3	6			1				2		12
llborn NOS irth Injury	5	2			1	2		2			12
cellaneous immaturity ardiopathy twin)	2	1									3
tal	12	13	2	1	5	5	1	3	5	3	50

pendently by each of us without knowledge of the clinical history or autopsy findings of the cases In 35 cases the primary classification of the lesions was uniform The ribs of the remaining 15 cases in which the primary judgements were disparate were reexamined and finally classified on open discussion The various types of CCJ lesions were then correlated to birth weight postnatal age and to other autopsy findings with particular reference to pulmonary disorders

Results

Slight to prominent abnormalities of the CCJ were recorded in 38 of the cases with a slight accumulation of cases with normal CCJ among the stillborn infants otherwise the type and degree of the CCJ lesion were not correlated to the postnatal age of the patient nor to the birth weight (Tables 1 and 2) However there was an apparent correlation between lesions of TR and IM types and the presence of pulmonary hyaline membranes (PHM) No less than 11 of the 12 PHM cases displayed these lesions (Table 1 Fig 1)

In 20 cases in which the CCJ lesion had been classified as TR or IM type a "reactive zone" was identified The size of this zone measured in the longitudinal axis of the rib varied from 500 to 1500 μ and was not correlated to the postnatal age of the patient (Table 2)

Comment

The present study confirms the findings by previous investigators (*Emery & Kalpaktsoglou* 1967 *von Sydow et al* 1956) in showing that CCJ abnormalities are frequent in cases of perinatal death

According to our opinion these lesions should not be regarded merely as a non specific manifestation of perinatal distress or placental insufficiency The various types of CCJ lesion rather indicate different aetiological factors possibly different specific deficiency states This was also considered by *von Sydow et al* (1956) who found alterations similar to rickets in their series of perinatal autopsy cases

control mice. After intervals varying from 2 to 21 days equal numbers of animals from each group were killed with chloroform. Two or three tissue specimens from each kidney were immediately frozen dried and treated with formaline vapour according to *Eranko* (1967) and *Corrodi & Jonsson* (1967). Pairs of slides were made from each specimen coded and examined independently by the two authors. The specific fluorescence of the sympathetic fibres and vesicles was examined in the arterioles and intralobular arteries in the interlobular arteries and in the arteriae arcuatae. The fluorescence was estimated subjectively as clear faint (= weak and occasional) or absent. Each animal gave rise to eight observations concerning each cathegory of vessels.

The most striking result was the high percentage without fluorescence in the arterioles and intralobular arteries of ascitic mice 58.8 ± 23.3 per cent as compared with 11.7 ± 10.3 per cent in the control group. The difference was statistically significant at the $p < 0.001$ level. The percentage of negative observations in interlobular arteries 20.6 ± 20.2 per cent also differed from the controls which were 5.3 ± 10.3 per cent but less significantly ($p < 0.01$).

The depletion of monoamines i.e. of noradrenaline (NA) seen in this experiment could be explained in various ways: possibly it is the result of an increased efferent activity in the sympathetic nervous system or it may be due to an ineffective biosynthesis or to an ineffective reuptake of NA in the nerve tissue or to a combination of these factors. NA is released from the adrenergic fibres and vesicles by means of electric stimulation of the sympathetic nerves in vivo and in vitro: the resulting decrease in these nerves was 6 to 37 per cent (*Malmfors* 1965 *Dearnaley & Geffen* 1966 *Fredholm & Sedvall* 1966 *Geffen* 1967 *Potter* 1967). 60 to 95 per cent of the amine released is recovered by the axons (*Brown* 1965 *Gillespie* 1966). In studies on drug induced depletion of the adrenergic transmitter and its subsequent reappearance it was found that the transmitter could be histochemically detected even if reduced by more than 90–95 per cent (*Andén et al* 1966 *Fuxe & Sedvall* 1964). The negative observations in this study therefore indicate other or further causative factors than increased nervous activity. *Malmfors* (1967) reported that chemical depolarization produced by exchanging sodium for potassium had a marked inhibiting effect on the accumulation of NA and that it also caused a very rapid disappearance of the fluorescence in the adrenergic fibres of rat irises *in vitro*. The ascitic mice probably exhibited secondary hyperaldosteronism with retention of sodium and depletion of potassium and this might hypothetically explain the depletion of monoamines.

Thickening and narrowing of the blood vessels was seen in kidney biopsies from normotensive patients with familial chloride diarrhea (*Pasternack & Perheentupa* 1967) and with the nephrotic syndrome (*Pasternack & Tallqvist* 1969). Both conditions are thought to be associated with secondary hyperaldosteronism due to hyperangiotensinism. We hope to elucidate the role of aldosterone and angiotensine in this context in further experiments.

References 1 *Andén N F Fuxe K Hamberger B & Hökfelt T* Acta Physiol Scand 67 306–312 1966 — 2 *Brown J* Proc Roy Soc (Biol) 162 1 13 1965 — 3 *Corrodi H & Jonsson G* J Histochem Cytochem 15 65–78 1967 — 4 *Dearnaley D P & Geffen L B* Proc Roy Soc (Biol) 166 303–315 1966 — 5 *Eränkö O* J Roy Micr Soc 87 259–276 1967 — 6 *Fredholm B & Sedvall G* Life Sci 5 2023–2032 1966 — 7 *Fuxe K & Sedvall G* Acta Physiol Scand 61 121 1 9 1964 — 8 *Geffen L B* In Catecholamines in Cardiovascular Physiology and Disease R Reader (Ed) The American Heart Association Inc New York 1967 pp 57 1 — 9 *Gillespie J S* Proc Roy Soc (Biol) 166 1–10 1966 — 10 *Malmfors T* Acta Physiol Scand 64 (suppl 248) 1–93 1965 — 11 *Malmfors T* In Catecholamines in Cardiovascular Physiology and Disease R Reader (Ed) The American Heart Association Inc, New York 1967 pp 25 49 — 12 *McKenna O C & Angelakos E T* Circ Res 22 345–354 1968 — 13 *Pasternack A & Perheentupa J* Lancet 1966 II 1047 1049 — 14 *Pasternack A & Tallqvist G* Acta Med Scand In press 1969 — 15 *Potter L T* In Catecholamines in Cardiovascular Physiology and Disease R Reader (Ed) The American Heart Association Inc New York pp 13 21 — 16 *Wager O & Räsänen J A* Ann Med Exp Biol Fenn 45 170 173 1967 — 17 *Veyrat R Muller A F & Mach R S* Presse Med 77 189 190 1969 — 18 *Wågermark J Ungerstedt U & Ljungqvist A* Circ Res 22 149 154 1968

ERRATA

Page 24 question five — myocardial infarction and diffuse fibrosis
read — myocardial infarction and cardiac ruptures
Page 26 par 4 line 1 parcentages read percentages
Page 27 notes concerning the myocardium line 9 ruptpres read ruptures
Page 30 par 4 line 2 towspeople read townspeople
Page .1 par 1 line 1 towspeople read townspeople

Pages 64—85	1			2			3			1	
	Mean value	No of cases	Statistical significance	No of cases	Mean value	Statistical significance	Mean value	No of cases	Statistical significance	No of cases	Mean value
OTH SEXES											
leart weight/body weight	6 8	315		180	7 7		7 3	433	P < 0 001	315	6 8
IALES											
leart weight/body weight	6 7	168		101	7 0		7 3	286	P < 0 001	168	6 7
'EMALES											
Ieart weight/body weight	6 9	147		79	8 7		7 4	147	P < 0 01	147	6 9

[ABLE .0

Page 67	1			2	
	No of cases	Mean value	Statistical significance	Mean value	No of cases
BOTH SEXES					
Heart weight/body weight	833	7 2		7 6	283
MALES					
Heart weight/body weight	54.	7 1	P < 0 01	7 6	174
FEMALES					
Heart weight/body weight	286	7 6		7 6	109

ABLE °1

Page 69 par 4 line 2 4? 5 % of 665 read 4? 5 % of 663
Page 0 par 2 line 4 town and country people read towns and country people
Page 76 par 3 line 10 males and females who dead read males and females who are dead
Page 7 par 3 line 6 arterior read anterior
Page 7 the last entence of the summary (Both in males ...) is to be deleted

Acta path microbiol scand 77 177–186 1969

The Department of Anatomy (Head Professor Lárus Einarson) and the Department of Otolaryngology (Head Professor H C Andersen) University of Aarhus Aarhus Denmark

DEOXYRIBONUCLEIC ACID CONTENT IN BRONCHOGENIC CARCINOMA WITH SPECIAL REFERENCE TO POLYPOID CELL NUCLEI

A Preliminary Report

By

O Greisen

Received 16 ix 69

Numerous studies of the deoxyribonucleic acid (DNA) content in tumours of various organs are available It is characteristic of the tumour cell nuclei that they have a high content of DNA and that the values show a wide dispersion In normal tissues the values are con centrated around a diploid mode, with a few nuclei within the range up to twice the diploid mode owing to the DNA synthesis prior to mitosis On the other hand in tumour tissue the values extend from a basal modal level with many cell nuclei in the range up to twice that level and often an appreciable number of values above the latter

Microspectrophotometric studies have been performed on various neoplasms including bronchogenic carcinoma (*Stowell* 1946 *Sandritter* 1952a *Leuchtenberger et al* 1954 *Boriani & Gandolfi* 1961 *Sandritter & Fisher* 1962 *Sandritter & Kleinhans* 1964 *Sandritter et al* 1958 *Sandritter et al* 1965 *Hahrer & Kaffenberger* 1962) Like other carcinomata the bronchogenic variety nearly always reveals a con siderably increased average content of DNA as compared with normal tissues and the values show an appreciable dispersion

As tumours differ from normal tissues in that they have a higher average content of DNA several authors have studied whether a rela tionship between the malignancy of a tumour and its DNA content should exist

However a number of factors must be considered when the average content of DNA in a tumour is to be determined

First owing to the wide dispersion of the DNA values in tumour tissue it is necessary to study a very large number of randomly selected cell nuclei Secondly the possible occurrence of necrosis—not only visible but also measurable (*Wust* 1960 *James* 1968)—must be taken into account Thirdly the average content of DNA will depend on the modal value of the tumour i e the value of the stem cell The modal

value will most frequently be within the hyperdiploid range but it may vary from hypodiploid to tetraploid or even higher

Bronchogenic carcinomata are very suitable objects for the study of the DNA content because the same organ harbours various histologically well defined types of carcinoma (*Kreyberg* 1967) whose course and degree of malignancy are well known from clinical observation

MATERIAL AND METHODS

The material studied was bronchial biopsy specimens obtained from patients who had been referred to the Department of Otolaryngology Aarhus Kommunehospital for bronchoscopy because of a suspected tumour The series comprised 13 cases of squamous cell carcinoma 8 cases of adenocarcinoma and 13 cases of small-celled anaplastic (oat cell) carcinoma None of the patients had received radiotherapy

The biopsy specimens were removed from the periphery of the tumour and at once fixed in 2 per cent formalin embedded in paraffin and cut into sections 7 μ in thickness The sections were treated with ribonuclease for 2 hours at 37 C in a phosphate buffer pH 7 6 and then stained with gallocyanin-chromalum (*Einarson* 1951) By this enzymatic treatment the massive cytoplasmic basophilia brought about by ribonucleic acid which is formed in the cell nucleus and diffuses into the cytoplasm (*Einarson* 1932 1933 1935) disappears completely while the nucleus assumes a dark blue colour against a bright background

The microspectrophotometric measurements were made by means of a Leitz spectrophotometer (*Einarson et al* 1965) The DNA concentration was calculated as the product of area and extinction and expressed in terms of arbitrary units (A U) Measurements were made on 30–40 nuclei from each tumour

Polyploid cell nuclei are defined as nuclei with a chromosome number larger than normal (*DeRobertis et al* 1965) With due allowance for premitotic DNA synthesis they may in microphotometry be defined as nuclei with a DNA content which is higher than twice the normal basal value The number of polyploid nuclei is stated per 1500–2000 tumour-cell nuclei

RESULTS

Fig 1 shows typical histograms of the distribution patterns of DNA in the population of nuclei in each of the three types of bronchogenic carcinoma studied

It is seen that the dispersion of the DNA content in the tumour cell nuclei is great—much greater than in the population of cell nuclei of normal bronchial tissue (*Greisen* 1968) and that the measurements tend to cluster around certain values of which the lowest basal one is the modal value of the tumour This is the DNA value for the stem cell of the tumour which is responsible for the tumour growth and which has a special modal chromosome number (*Makino* 1957)

Another peak is seen at twice the modal value This is largely due to cell which have synthetised DNA in preparation of mitosis There are many intermediate values which are due to cells synthetizing DNA prior to mitosis or to cells with an aneuploid DNA content The average DNA content in the three types of bronchogenic carcinoma was much higher than that of normal bronchial epithelium (*Greisen* unpublished data) There was no difference in the modal values in the three types (Table 1) their basal modal values ranging within the groups near

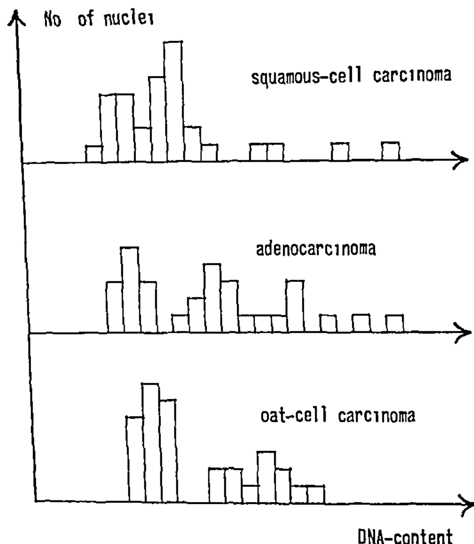


Fig 1

Histograms showing the distribution patterns of DNA in cell nuclei of bronchogenic carcinomata

diploid 12 ± 3 A U near triploid 18 ± 3 A U and near tetraploid 24 ± 3 A U (*Wakonig Vaartaja & Hughes* 1967)

From the measurements made it appeared that the average DNA concentration was slightly lower in the oat cell carcinoma than in the other two groups also after correction for incipient necrosis if any As is seen from Table 1 there was no difference in the modal values in the various groups which could explain this lower average On the other hand the measurements suggested that the distribution patterns



of the nuclei of the three types of carcinoma differred with only a small number of cells with a high DNA content in the oat cell carcinoma In order to study this phenomenon in greater detail measurements were performed in tumour sections on all nuclei which seemed to have a DNA content which was close to or above twice the modal value per 1500–2000 cell nuclei The DNA content of these nuclei was calculated if the figures thus obtained were above twice the basal modal value + 2 5 S D the nuclei were recorded as polyploid (Fig 2) The average number of polyploid nuclei appears from Table 2

TABLE 1

Modal Values of the Three Groups of Carcinoma

Type	Near diploid	Near triploid	Near tetraploid
Squamous cell carcinoma	6	7	0
Adenocarcinoma	5	3	0
Oat cell carcinoma	5	5	0

It is seen that the number of polyploid nuclei in the squamous cell carcinoma and adenocarcinoma is about 10 times as large as in the oat cell carcinoma

TABLE 2

Average Number of Polyploid Nuclei per 1000 Cells

Type	Per 1000 cells
Squamous cell carcinoma	5 9
Adenocarcinoma	6 7
Oat cell carcinoma	0 6

Fig 3 shows the DNA content of polyploid nuclei per 1000 nuclei in each tumour section It appears that most sections of oat cell carcinoma contained from 0 to 1 polyploid nuclei per 1000 while the number in the other two types was appreciably higher in most cases and that there was only a slight degree of overlapping

In the polyploid nuclei of the oat cell carcinoma the DNA concentration was relatively close to twice the modal value whereas the squamous cell carcinoma and adenocarcinoma contained many nuclei with a very high content of DNA up to 10–11 times as much as the basal modal value

---

Fig 2

Photomicrographs showing polyploid nuclei of squamous cell carcinoma adenocarcinoma and for comparison the more uniform appearance of oat cell carcinoma (× 250)

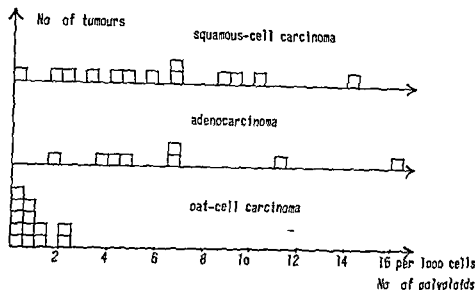


Fig 3
Number of polyploid nuclei in the three types of carcinoma

TABLE 3
Number of Polyploid Nuclei and Their DNA Content in Relation to the Modal Value

DNA content/ modal value	Squamous cell carcinoma	Adeno carcinoma	Oat-cell carcinoma
2 5– 2 9	40	35	7
3 0– 3 4	30	18	6
3 5– 3 9	10	18	2
4 0– 4 4	12	6	–
4 5– 4 9	8	4	1
5 0– 5 4	3	1	–
5 5– 5 9	7	5	–
6 0– 6 4	2	1	–
6 5– 6 9	2	3	–
7 0– 7 4	1	2	–
7 5– 7 9	1	–	–
8 0– 8 4	1	1	–
8 5– 8 9	–	–	
9 0– 9 4	1	–	–
9 5– 9 9	–	–	
10 0–10 4	–		
10 5–10 9	–	–	–
11 0–11 4	–		
11 5–11 9	1	–	–

DISCUSSION

Among the three most common bronchogenic carcinomata the prognosis is reported to be most favourable for the squamous cell variety just as good or nearly as good for adenocarcinoma but much graver for oat cell carcinoma (*Karlkin et al* 1955 *Boucot et al* 1959 *Goldman* 1965 *Hyde et al* 196› *Magnues* 1966 *Jones et al* 1967)

It is characteristic of carcinomata that on an average the DNA content is increased as compared with that of nuclei of normal tissue A number of authors (*Sandritter* 1952a b *Stich et al* 1960 *Sandritter & Kleinhans* 1964 *Jacobsen* 1968) have studied various types of neoplasms in order to ascertain whether there is a relationship between the average content of DNA on the one hand and the malignancy of the tumour and the prognosis on the other but they did not find any evidence in support of this assumption

Atkin and his associates made an attempt to correlate the basal modal value of the DNA content of a tumour and its histological differentiation and the prognosis In the series of 165 cases of carcinoma of the uterus *Atkin et al* (1959) did not reveal any correlation between the basal modal value and the survival time of the patients In subsequent series they demonstrated a significantly better prognosis for tumours with modal values within the tetraploid range while tumours with modal values within the diploid range had a poorer prognosis (*Atkin & Richards* 1962 *Atkin* 1964)

In the present series of bronchogenic carcinomata there was no difference in the modal values in the three groups and they revealed only a slight difference in the average DNA content On the other hand there were 10 times as many polyploid nuclei in squamous cell carcinoma and adenocarcinoma as in oat cell carcinoma In addition the polyploid nuclei in the first two groups had a much higher DNA content than the last group

Under normal conditions polyploid nuclei occur in certain human tissues such as bone marrow (megakaryocytes) and the liver (*Hauschka* 1963) Polyploid nuclei have also been observed in various organs of insects and other animals (*Swift* 1950) According to the literature and my own investigations polyploid nuclei are absent in normal bronchial epithelium

The formation of polyploid nuclei may occur after the synthesis of DNA by an inhibition of the splitting of the chromosomes or of their movements (*Levan & Hauschka* 1953 *Gelfant* 1963) Another mode of formation of polyploid nuclei was demonstrated by *Robberts & Cole* (1964) who found that these nuclei could form in ascites tumour cells by fusion of two unicellular nuclei during mitosis

In tissue culture unfavourable growth conditions may give rise to a shift from diploid to tetraploid cells possibly due to the formation of certain substances in the nutrient medium which inhibit cytokinesis or to which tetraploid cells are more resistent than diploids (*Hsu* 1961) Such transformations were observed in *in vitro* experiments by *Levan & Biesele* (1958) and *Hsu & Klatt* (1959)

Provided that the vital processes are preserved a tetraploid cell is from a theoretical point of view only half as efficient as a diploid cell in cell reproduction In order to divide once the former must synthetize twice as much material as the latter (*Hsu* 1961) This was shown ex

perimentally by *Hauschka et al* (1957) in the case of two ascites tumours of identical genetic constitution In three mouse ascites tumours isolated from the same highly virulent ascites lymphosarcoma strain *Hauschka et al* (1956) found that the cells from the tetraploid strain in spite of slight iso antigenic effect were less virulent less invasive and required longer time to kill the animals than the more virulent diploid strains

When polyploid nuclei are much more frequent in squamous cell carcinoma and adenocarcinoma which have a better prognosis than the highly virulent oat cell carcinoma this may mean that the two former tumour types provide less favourable growth conditions causing inhibition of mitosis while the cells continue to synthetize DNA resulting in the formation of these large dark nuclei with a high content of DNA

The considerably greater malignancy of the oat cell carcinoma is due to its greater tendency to rapid growth and rapid metastatic spread and to its invasion of the pulmonary vessels (*Kreyberg* 1962 *Nohl* 1962 *Collier* 1957)

If the polyploid cells can be taken as a manifestation of unfavourable growth conditions and a lower virulence it must be assumed that only relatively slight inhibition of the growth occurs in the oat cell carcinoma as compared with squamous cell carcinoma and adenocarcinoma which is in good agreement with clinical observations

SUMMARY

The DNA content of bronchogenic carcinomata was measured by a microspectrophotometric technique It was found that the number of polyploid tumour cell nuclei was 10 times as large in squamous cell carcinoma and adenocarcinoma as in oat cell carcinoma

The formation of the polyploid nuclei and their role in malignancy are discussed

REFERENCES

Atkin N B Nuclear size in carcinoma of the cervix its relation to DNA content and to prognosis Cancer *17* 1391–1399 1964

Atkin N B & *Richards B M* Clinical significance of ploidy in carcinoma of cervix its relation to prognosis Brit Med J *2* 1445–1446 1962

Atkin N B Richards B M & Ross A Deoxyribonucleic acid content of carcinoma of uterus assessment of its possible significance in relation to histopathology and clinical course based on data from 165 cases Brit J Cancer *13* 773 787 1959

Boriani A & *Candolfi M* Osservazioni istochimiche quantitative sull acido desossiribonucleico dell epitelio bronchiale nelle bronchiti croniche e delle neoplasie bronchopolmonari Arch Ital Otol *72* 678 640 1961

Boucot K R Horie L & Sokoloff M J Survival in lung cancer New Engl J Med *260* 742 1959

Collier F C Blakemore W S Kyle R H Enterline H T Kirby C K & Johnson J Carcinoma of the lung factors which influence five years survival with special reference to blood vessel invasion Ann Surg *146* 417 423 1957

DeRobertis E D P Nowinski W W & Saez F A Cell Biology Saunders Company Philadelphia and London 1965

Einarson L A method for progressive selective staining of Nissl and nuclear substance in nerve cells Amer J Path *8* 295 307 1932

Einarson L Notes on the morphology of the chromophil material of nerve cells and its relation to nuclear substances Amer J Anat *53* 141–175 1933

Einarson L Histological analysis of the Nissl pattern and substance of nerve cells J Comp Neurol *61* 101–133 1935

Einarson L On the theory of gallocyanin chromalum staining and its application for quantitative estimation of basophilia A selective staining of exquisite progressivity Acta path microbiol scand 28 82–102 1951

Einarson L Hansen F & Kruger H G Das elektrische Leitz mikroskop photometer MPF und seine Anwendung in der Cytophotometrie Leitz Mitteilungen für Wissenschaft und Technik 3 129–134 1965

Gelfant S Inhibition of cell division a critical and experimental analysis Int Rev Cytol *14* 1–39 1963

Goldmand K P Histology of lung cancer in relation to prognosis Thorax 20 298–307 1965

Greisen O Mitotic activity assessed by a new method and nucleic acid content of the bronchial epithelium Les Bronches in press 1968

Hauschka T S Chromosome patterns in primary neoplasia Exp Cell Res suppl *9* 86 98 1963

Hauschka T S Grinnell S T Révész L & Klein G Quantitative studies on the multiplication of neoplastic cells *in vivo* Influence of doubled chromosome number on growth rate and final population size J Nat Cancer Inst *19* 13–31 1957

Hauschka T S Kvedar B J Grinnell S T & Amos D B Immunoselection of polyploids from predominantly diploid cell populations Ann New York Acad Sci 63 633–705 1956

Hsu T C Chromosomal evolution in cell populations Int Rev Cytol *12* 69–161 1961

Hsu T C & Klatt O Mammalian chromosomes *in vitro* Heteroploid transformation in neoplastic cells J Nat Cancer Inst 22 313–339 1959

Hyde L Yee J Wilson R & Patno M F Cell type and the natural history of lung cancer J A M A 193 52–54 1965

Höhrer H & Kaffenberger H Vergleichende UV photometrische Nucleinsäure bestimmungen an normalen und Tumorzellen des Menschen Ann Histochim suppl 2 19–23 1962

James J Feulgen DNA changes in Rat liver cell nuclei during the early phase of ischaemic necrosis Histochemie *13* 312 322 1968

Jacobsen A E Prostataepithelkerners relative nucleinsyreindhold Munksgaard 1968

Jones J C Kern W H Chapman N D Meyer B W & Lindesmith G G Long term survival after surgical resection for bronchogenic carcinoma J Thoracic & Cardiovasc Surg 54 383 391 1967

Kirklin J W McDonald J R Clagett O T Moersch H J & Gage R P Bronchogenic carcinoma cell type and other factors relating to progress Surg Gynec & Obst *100* 429–438 1955

Kreyberg L Histological lung cancer types Norwegian Universities Press Oslo 1962

Kreyberg L Histological typing of lung tumours World Health Organization Geneva 1967

Leuchtenberger C Leuchtenberger R & Davis A M Microspectrophotometric study of the desoxyribose nucleic acid (DNA) content in cells of normal and malignant human tissues Am J Pathol *30* 65–85 1954

Levan A & Biesele J J Role of chromosomes in carcinogenesis as studied in serial tissue culture of mammalian cells Ann N Y Acad Sci *71* 1022–1053 1958

Levan A & Hauschka T S Endomitotic reduplication mechanism in ascites tumors of the mouse J Nat Cancer Inst *14* 1–43 1953

Maamies T J Bronchogenic carcinoma Diss Helsinki 1966

Makino S The chromosome cytology of the ascites tumors of rats with special reference to the concept of the stemline cell Int Rev Cytol *6* 25–84 1957

Nohl H C The spread of carcinoma of the bronchus Lloyd Luke London 1962
Robberts D C & Cole G F Some mechanics of formation of polyploid and heteroploid cells in murine ascites tumor *in vitro* J Nat Cancer Inst 32 1023-1030 1964
Sandritter W Über den Nucleinsäurestoffwechsel in Plattenepithel - und kleinzelligen Bronchialkarzinomen Frankfurt Z Path 63 387-422 1952 a
Sandritter W Über den Nucleinsäuregehalt verschiedenen Tumoren Frankfurt Z Path 63 423-446 1952 b
Sandritter W & Fischer R Der DNS Gehalt des normalen Plattenepithels des carcinoma in situ und des invasiven Carcinoms der Portio Proc of the First Int Congr of Exfoliative Cytol (Wied G L Ed) 189-194 Lippincott Comp Philadelphia 1962
Sandritter W & Kleinhans D Über das Trockengewicht den DNS und histonproteingehalt vom menschlichen Tumoren Z Krebsforsch 66 333-348 1964
Sandritter W Muller W Schäfer D H & Schiemer H G Histochemie von Spulumzellen Quantitative ultraviolette mikrophotometrische Nucleinsäurebestimmungen Frankfurt Z Pathol 68 710-727 1958
Sandritter W Seidel A Kleinhans D Paddags I & Dontenwill W Cytophotometrische Messungen des DNS Gehaltes an menschlichen und tierexperimentellen Bronchialmetaplasien Z Krebsforsch 67 69-79 1965
Stich H F Florian S F & Emson H E The DNA content of tumor cells Polyps and adenocarcinomas of the large intestine of man J Nat Cancer Inst 24 471-482 1960
Stowell R F Nucleic acids in human tumors Cancer Research 6 426-435 1946
Swift H F The deoxyribose nucleic acid content of animal nucleic Physiol Zool 23 169-198 1950
Wakonig Vaartaja R & Hughes D T Chromosome studies in 36 gynaecological tumours of the cervix corpus uteri ovary vagina and vulva Eur p J Cancer 5 263-277 1967
Wüst G Über den Nucleinsäuregehalt bösartiger menschlichen Tumoren und ihrer Metastasen Arch Geschwulstforsch 16 324 335 1960

Acta path microbiol scand 77 187-195 1969

Second Department of Medicine and Laboratory of Electron Microscopy
University of Helsinki Finland

ULTRASTRUCTURE OF VARIOUS METAPLASIAS OF THE STOMACH

By

S Tarpila A Telkka and M Siurala

Received 20 iii 69

In atrophic gastritis normal body glands are replaced by foreign ones 1) intestinal type 2) antral type (pseudo pyloric metaplasia of Schindler) 3) Brunner type and other less well characterized types (13) The present paper is concerned with electron microscopy of intestinal and pseudopyloric metaplasias of the gastric body mucosa Preliminary reports of this study have been published elsewhere (17 18)

MATERIAL AND METHODS

Biopsy specimens were obtained by means of suction biopsy tube from eight patients with intestinal metaplasia, from five patients with pseudopyloric metaplasia and from two patients with both intestinal and pseudopyloric metaplasia in the specimens For the purpose of comparison biopsy specimens were obtained from normal gastric mucosa (two patients) from normal antral mucosa (three patients) and from normal jejunal mucosa (five patients) Antral biopsies were obtained 3-4 cm proximal to the pylorus These specimens showed by light microscopy an uniformly normal antral mucosa In the group of 15 patients with atrophic gastritis four were suffering from pernicious anaemia

The specimens were immediately divided into two parts One was fixed in 10 per cent neutral formalin stained with haematoxylin eosin and examined by light microscopy The other was first fixed in glutaraldehyde and then in osmium tetroxide Dehydration was performed in a series of alcohol solutions ranging from 50 per cent to obsolute alcohol Thereafter the specimens were embedded in Epon resin (8) and sectioned with a glass knife in a Porter Blum microtome Electron microscopic examination was performed with a Siemens Elmiskop I or with Philips EM 200

RESULTS

Normal Gastric Body Mucosa

Using ordinary stains the main epithelial cell types seen in light microscopy are 1) surface epithelial cells 2) mucous neck cells 3) zymogenic cells 4) parietal (oxyntic) cells 5) argentophil cells Their ultrastructure has been studied in animals (3 4 5) and in man (6 7)

Requests for reprints should be addressed to Simo Tarpila Second Department of Medicine Meilahti Hospital Haartmanink 4 Helsinki 29

This study was aided by a grant from *Sigrid Juselius Foundation*

For the purpose of comparison the main features of the ultrastructure of these cells will be given below

There are two types of mucous cells in the gastric mucosa 1) *surface mucous cells* and 2) *mucous neck cells* The granules of these cells have an appearance different from that of the mucous granules seen in any other tissue The gastric mucus is very dense and stippled instead of having the pale homogeneous appearance seen in the goblet cells of the small intestine The stippled appearance is most intense in the surface mucous cells Likewise the density of granules is greatest in the surface mucous cells The granules of the mucous neck cells are pale and very often hemilunar shaped

3) *Parietal or oxyntic cells* are characterized by intracellular canaliculi large numbers of mitochondria and microvilli which cover the border of the cells 4) *Zymogenic cells* contain large pale homogeneous granules and a very dense network of granular endoplasmatic reticulum at the base of the cell 5) *Argentophil cells* which are located near the basement membrane contain small dark granules

Normal Jejunal Epithelium

In the following only the ultrastructure of the striated border cells and goblet cells of the jejunal mucosa will be described For details reference should be made to the study of *Palay & Karlin (10)*

The microvilli of the absorptive cells are long and uniform The nuclei are prolonged and located basally There are large numbers of mitochondria and a dense network of granular and smooth endoplasmatic reticulum *A few lysosomes are seen Below the microvilli is* the terminal web

The goblet cells are oval and filled with pale homogeneous mucous granules

---

Fig 1

Intestinal metaplasia of the body mucosa

1a Light microscopical picture of intestinal metaplasia Only intestinal type of glands is visible H E, × 75

1b *Typical* absorptive" cell with microvilli plenty of mitochondria (M lysosome (Ly) well developed endoplasmatic reticulum (ER) terminal web TW) and desmosomes (D) × 31 000

1c Typical goblet cell filled with mucous granules (C) × 18 000

1d A cell with rather well developed microvilli but with numerous mucous granules (G) × 30 000

1e The *numerous mucous* granules of hemilunar shape resemble those seen in normal mucous neck cells of the body mucosa × 42 000

1f Intestinal type of cell which has less well developed *microvilli and terminal* web than those seen in Fig 1b resembling crypt cells of true intestinal mucosa × 4,500

Intestinal Metaplasia of the Stomach

In specimens obtained from areas of the body with light microscopic signs of intestinal metaplasia many cells were seen which closely resembled *the absorptive cells* of normal jejunal mucosa (Fig 1b) The microvilli are long (1–1 5 μ) uniform and located close to one another Their central filaments are thin and smooth, as in true intestinal microvilli There is an almost structureless terminal web and below it numerous mitochondria a smooth and granular endoplasmatic reti



culum and lysosomes However in some of these cells of intestinal type the microvilli and terminal web are less well developed and lysosomes are almost lacking They resemble *cells of the normal intestinal crypts* rather than those of the villi (Fig 1f)

Numerous cells similar to *the goblet cells* of the normal intestinal epithelium are seen in the metaplastic tubules of the gastric mucosa (Fig 1c) On the other hand, among the typical goblet and absorptive cells others are found which are not present in the normal intestinal epithelium (Figs 1d e) Fig 1d shows a mucus secreting cell which resembles cells of the surface and pits of a normal gastric mucosa in that they have numerous mucous granules a poorly developed endoplasmatic reticulum and only a few mitochondria However the microvilli of these cells are numerous and better developed than those in the normal gastric surface epithelial cells In Fig 1e a type of cells is shown which is commonly found in the more superficial parts of the intestinal metaplasia These cells contain dense granules many of which have a hemilunar appearance of the granules of the *mucous neck cells* of the normal body mucosa

It appears that the ultrastructure of the intestinal metaplasia although it resembles that of true intestinal epithelium is not as uniform the latter among typical intestinal cells others are seen which resemble normal elements of a body mucosa rather than those of a true intestinal epithelium

Normal Antral Mucosa

Fig 2 shows the various cell types found in normal looking antral mucosa in light microscopy (Fig 2a) Most cells contain large amounts of dense granules Endoplasmatic reticulum and mitochondria are scanty (Fig 2b) The nucleus is elongated and located at the base of the cell On the hole these cells are similar to those of the surface and pits of the normal body mucosa These cells occupy the more superficial parts of the tubules Among these cells are others with paler and

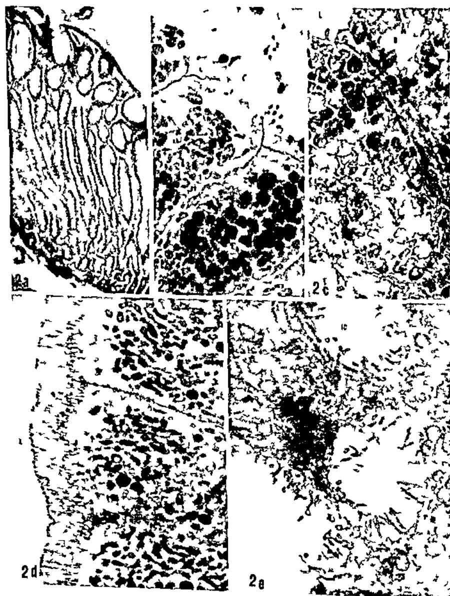
---

Fig 2

Normal antral mucosa

2a Light microscopical picture of normal antral mucosa H E, × 75
2b Surface cells filled with dense granules *similar to the surface cells of the body* mucosa × 5400
2c Surface cells with pale granules (G) resembling somewhat the mucous neck cells of the body mucosa × 5400
2d Cells with all characteristics *of normal intestinal* absorptive cells × 5400
2e A cell of a deeper part of the gland with intracellular canaliculi (IC) *typical of* parietal cells In *addition numerous* mitochondria are present × 8400

smoother granules a better developed endoplasmatic reticulum with ribosomes and more mitochondria (Fig 2c) These cells greatly re semble the mucous neck cells of the body mucosa however the hemi lunar shaped granules characteristic of the latter are lacking In the deeper parts of the tubule in addition to the above mentioned types are cells which cannot be distinguished from normal absorptive in testinal cells (Fig 2d) and cells containing canaliculi of the same



The normal antral mucosa contained cells some of which closely resembled normal absorptive intestinal cells while others showed characteristics of parietal cells. It seems as if the normal antral mucosa is capable of producing different cell types normally present in the adjacent mucosal structures. This suggests a large spectrum of differentiation capacity of the germinative centres of the gastric mucosa and is in agreement with the findings of *Ming et al* (9). These multipotent characteristics may explain the development of intestinal and antral types of glands in connection with gastritis and the common occurrence of tubules resembling intestinal and antral ones in the body mucosa of embryos. This differentiation capacity of germinative centres seems to be maintained to some degree even in very advanced stages of atrophic gastritis as shown by regeneration of parietal cells during treatment with corticosteroids (15).

SUMMARY

The ultrastructure of intestinal and pseudopyloric metaplasia of the stomach is studied and compared with that of the normal antral body and jejunal mucosa.

Electron microscopy revealed that normal antral tubules in spite of their uniformity under the light microscope contained various cell types: 1) cells resembling normal surface and foveolar cells of the body mucosa, 2) cells similar to normal jejunal absorptive cells, 3) cells resembling mucous neck cells of a body mucosa and 4) cells with some characteristics of parietal cells corresponding to the white parietal cells described by *Lillibridge*. Goblet cells were not seen. This suggests a multipotential differentiation capacity of the germinative centres of the gastric mucosa.

Pseudopyloric metaplasia of the body mucosa contained the same types of cells as normal antral epithelium. However parietal like cells were lacking and the remaining cell types were less well identified with normal intestinal cells and cells of the body mucosa than the various cells found in a normal antral epithelium.

Intestinal metaplasia of the body mucosa contained cells which were similar to goblet cells and absorptive cells of normal jejunal epithelium. However in the more superficial parts of the same tubules were other cells some of which resembled superficial cells while others resembled mucous neck cells of the body. In spite of the uniformity found by light microscopy it appears that intestinal metaplasia contains cell types which are not normally found in jejunal mucosa.

REFERENCES

1. *Demling L., Günther F. C., Teubner A.:* Zur Ultrastruktur der menschlichen Magenparenchymzellen bei Gastritis. Gastroenterologia [illegible] 145-149, 196[illegible]
2. *Elliot R. L. & Cullen R.:* Gastric biopsies. An ultrastructural study with special reference to pernicious anemia. Arch. Path. 77: 258-67, 196[illegible]

3 *Helander H F* Ultrastructure of fundus glands of the mouse gastric mucosa J Ultrastruct Res *Suppl 4* 1–123 1962
4 *Helander H F* Ultrastructure of secretory cells in the pyloric gland area of the mouse gastric mucosa J Ultrastruct Res *10* 145–159 1964
5 *Ito S & Winchester R* The fine structure of the gastric mucosa in the bat J Cell Biol *16* 541–577 19f3
6 *Lillibridge C B* The fine structure of normal human gastric mucosa Gastroenterology 47 269–290 1964
7 *Lillibridge C B* Electron microscopy of human gastric mucosa Progress in Gastroenterology Glass I B J Grune & Stratton *1* 2? 31 19f8
8 *Luft J H* Improvements in epoxy resin embedding methods J Biophys Biochem Cytol *9* 409–414 1961
9 *Ming S C Goldman H & Freiman D G* Intestinal metaplasia and histogenesis of carcinoma in human stomach light and electron microscopic study Cancer *20* 1418–14?9 1967
10 *Palay S L & Karlin J L* An electron microscopic study of the intestinal villus I The fasting animal J Biophys Biochem Cytol *5* 363–37? 1959
11 *Rubin W Ross L L Jeffries G H & Sleisenger M H* Intestinal heterotopia A fine structural study Lab Invest *15* 10?4–1049 1966
12 *Shiao Fu Chiao Weisberg H & Glass B B J* Ultrastructure of the gastric mucosa in atrophic gastritis and gastric atrophy Gastroenterology *54* 1??5 1968
13 *Siurala M* Pathology of atrophic gastritis Ann Med Int Fenn 4J 67–90 1956
14 *Siurala M & Launiala K* Disaccharidase activities of the atrophic gastric mucosa with intestinal metaplasia Scand J Gastroent 3 160–16? 1968
15 *Siurala M Peltola P & Varis K* The effect of long term treatment with prednisone upon the state of the human gastric mucosa S and J Gastroent 3 407–41? 1968
16 *Siurala M & Tarpila S* Absorptive function of intestinal metaplasia of the stomach Scand J Gastroent *3* 76–79 1968
17 *Tarpila S Telkka A & Siurala M* Electron microscopic structure of various metaplasias of the stomach Scand J clin Lab Invest ?1 suppl 101 77 1968
18 *Tarpila S Telkka A & Siurala M* Electron microscopic structure of intestinal metaplasia of the stomach Scand J clin Lab Invest *19* suppl 95 71 1967
19 *Tichy J & Hradsky M* Ultrastructure of the gastric mucosa in healthy human subjects and in patients with perni ious anemia Gastroenterologia *107* 79–388 1967

Acta path microbiol scand 77 196–200 1969

The Department of Pathology, University of Cambridge, England

PROTECTION OF C3H MICE AGAINST BP8 TUMOUR BY RNA EXTRACTED FROM LYMPH NODES AND SPLEENS OF SPECIFICALLY SENSITIZED MICE

Preliminary Communication

By

C. T. C. KENNEDY, D. B. CATER and I. HARTVEIT

Received 28 III 69

In many different immunological systems transfer of the capacity to evoke a heightened response to a specific antigen has been achieved using cells of the lymphocyte series with and without macrophages and less frequently with macrophages alone (*Gowans & McGregor* 1965, *Wilson & Billingham* 1967, *Bloom & Chase* 1967).

Cater & Waldmann (1967) have previously shown that intraperitoneal inoculation of lymph node cells from C57Bl mice immunized with BP8 ascitic tumour cells emulsified in Freund's complete adjuvant gave complete protection to C3H mice against simultaneous intraperitoneal challenge with a lethal dose of the tumour. The lymph node cells would protect if given intravenously and also if they were X irradiated with 500 rads (*Root & Cater* 1968). This suggested that a subcellular fraction may be involved, that the cells were transferring 'information' to the challenged mice.

Many investigations have been carried out using cell free extracts (usually RNA) from lymph nodes, spleens, macrophages, circulating lymphocytes etc. to effect the same type of transfer *in vivo* or elicit immunological responses *in vitro* (*Dutton* 1967). These responses are often much smaller than those initiated by intact cells, and because the BP8 tumour given intraperitoneally is always lethal to C3H mice, this syngeneic system was thought to provide a rigorous test of the effectiveness of a cell free extract.

Although the work is still at a preliminary stage we feel that the results obtained so far are of interest and certain of our findings, in particular the RNA extraction procedure, may be useful to others working in this field.

Gibb Fellow of the *British Empire Cancer Campaign for Research*.
Visiting guest worker, Research Fellow, *Norwegian Cancer Society*, The Gade Institute, Department of Pathology, University of Bergen, Norway.

MATERIAL AND METHODS

C57Bl mice were immunized 5 times at weekly intervals by injection of an emulsion of BP8 cells in Freund s complete adjuvant into 4 subcutaneous sites (*Cater & Waldmann* 1967) Non tumour control RNA was produced by injecting Ca/Bl mice with freeze pressed C3H liver and spleen emulsified in Freund s complete adjuvant

Six days after the last injection lymph nodes and spleens were harvested at +4 C, and used either to prepare lymphocyte suspensions or RNA

Various methods were used for *RNA extraction* but the most satisfactory was a modification of *Scherrer & Darnell s* method (1962) This gave the highest yield with the least degradation as judged from sucrose density gradient profiles or contamination with DNA (less than 5 per cent) and protein (less than 1 per cent) Our modifications of this 60 C phenol SDS procedure were (1) the organs were placed in Tyrodes solution containing 1 mg/ml PVS (K salt of Polyvinyl sulphuric acid supplied by Eastman Kodak) until all were harvested (2) PVS was used at 0 1 mg/ml during homogenization This procedure was followed as it was found using the method of *Roth* (1967) that with RNA at 1 mg/ml 0 1 mg/ml PVS would almost completely inhibit the production of acid insoluble RNA breakdown products by 10 1 μg/ml RNAase (bovine pancreatic Sigma Chemical Co Type XII A) Estimations of RNAase in 1 ml mouse serum and approx 10^7 disintegrated lymph node cells by the same method indicated levels of 8 × 10 μg RNAase in each sample

The RNA was injected into the footpads of the C3H mice in 0 1 ml phosphate buffered saline (pH 6 9) containing PVS at 0 1 mg/ml To check that the cells from which the RNA was extracted were active one group of mice per experiment was protected intraperitoneally with approx 2 × 10 living lymph node cells from immunized C57Bl mice Challenge was made with 2 × 10^4 or 10^3 living BP8 cells intraperitoneally one day or 5 days after the test injections Effects of the materials tested were followed by charting fractional weight change subjective scoring of *abdominal size and survival*

RESULTS

When *challenged with BP8 tumor cells* and given no protection all mice died Survival was 11 to 20 days mean 16 24 ± 0 29 days (n = 21)

Anti BP8 RNA when given at the optimum dose (50–200 μg/mouse) and at the optimum time *i e* the day before challenge resulted in 4 mice with complete protection and 41 mice which lived 16 to 32 *days after challenge mean survival 19 32 ± 0 43* days *(n = 41)* This was significantly longer than the controls t = 5 89 n = 60 P<0 001

The mice which showed complete protection developed a full blown ascites which lasted until the 20th to 25th day and then quite suddenly over the course of 2 or 3 days the ascites disappeared These completely protected mice also survived rechallenge see below

Larger doses of Anti BP8 RNA (300 to 1000μg/mouse) were not so effective There were no long term survivors Survival ranged from 17 to 22 days with a mean of 18 93 ± 0 4 (n = 15) This is still significantly longer than the controls t = 2 75 n = 29 P<0 01 but is not as good as the smaller doses *Large doses (500 to 1000 μg)* were also given to 6 mice 5 days before challenge with survival times of 17 to 19 days mean 18 3 ± 0 46 This was not significantly longer than their controls (t = 1 8 n = 20 P<0 1)

If a dose of 50 to 200 μg/mouse was split and half given the day before challenge and half given 4 days after the first there was even

better protection *i e* in 9 mice including one with complete protection (killed on the 107th day) the mean survival was 30 3 ± 9 7 (n = 9) compared with 19 16 ± 0 5 and no long term survivors in 19 mice given the single dose

Non tumour control RNA, in doses of 50 to 200 μg/mouse slightly delayed the onset of irreversible abdominal swelling and increased the survival time slightly 18 7 ± 0 7 (n = 14) significant at the 5 per cent level With larger doses (300 to 1000 μg/mouse) the mean survival time of 18 2 ± 0 6 (n = 13) was not significantly increased compared with their controls

With rat spleen RNA the survival time (13 75 ± 0 5 n = 4) was less than in the untreated controls

"Anti BP8" intact lymph node cells gave complete protection with negligible abdominal swelling Following *rechallenge* of the long term survivors with 5 × 10⁴ living BP8 cells on day 74 the lymph node cell protected group all died in 13 to 15 days but those that had received immune RNA were again long term survivors This time there was no initial abdominal swelling

No protection was afforded by 2 × 10⁷ C3H lymphocytes incubated with anti BP8 RNA *in vitro* for 30 minutes and then injected intraperitoneally into C3H mice either 5 days or 1 day before challenge

DISCUSSION

The present preliminary experiments show that protection can be afforded against tumour growth in a syngeneic system by the injection of RNA extracted from lymphocytes from specifically sensitized mice There appears to be an optimal time for injection *i e* the day before tumour challenge or the day before and four days later There also appears to be an optimal dosage *i e* 50 to 200 μg/mouse larger doses being ineffective Further work is however needed to clarify this effect

It was remarkable that the mice that finally showed protection initially developed a full blown ascites which suddenly disappeared about 3 weeks after challenge In an extensive experience with the BP8 tumour we have never seen this happen before These completely protected mice also survived rechallenge this time without initial ascites formation in contrast to the mice that had been protected initially with lymph node cells The underlying pathology here too needs further investigation

The effect of degradation of RNA was marked In the experiments reported showing protection by Anti BP8 RNA sucrose density gradient profiles showed that the RNA was undegraded Other parallel experiments in which RNA showing degradation was used showed poor protection In the reported experiments PVS was used to inhibit RNAase and this gave better results than bentonite or protamine sulphate Also RNA with less that 3 per cent DNA and 1 per cent protein

appeared to be better than samples containing more of these impurities A sample stored at –20 C in ethanol for 1 month remained active It has been suggested by *Krechetova et al* (1963) that PVS forms a loose complex with RNA probably a factor of importance in the RNAase inhibiting activity of PVS

Alexander et al (1967) have reported experiments demonstrating that RNA extracted from lymphoid cells of specifically sensitized animals can halt the growth of a solid tumour when injected near the site of implantation However their RNA extract contained up to 50 per cent DNA and it has been shown that exogenous DNA can induce tumour regression (*Glick & Goldberg* 1966) In view of the important implications arising from *Alexander's* work it is clearly necessary to examine further the role and nature of the RNA effective in this type of transfer experiment Since the RNA prepared by our method has less than 5 per cent DNA less than 1 per cent protein is relatively undegraded and is injected at sites distant from the tumour it is likely that this system will provide a new approach to the study of immunological control of tumour growth Among problems to be examined are 1) the conditions for complete protection 2) the specificity of the immunological protection 3) which definable components of the RNA extract are required for protection 4) reasons for the apparent optimum dosage and 5) the role of PVS

SUMMARY

C57Bl mice were immunized against BP8 tumour cells emulsified in Freund's complete adjuvant Either lymph node cells or RNA extracted from lymph nodes and spleens of the animals were used to protect C3H mice against a fatal challenge by the syngeneic BP8 tumour inoculated intraperitoneally The RNA was effective when injected into the footpads and given in optimal dosage

These results were obtained when a hot phenol method was used to extract the RNA which was then analysed for RNA DNA and protein Sedimentation profiles were produced to evaluate degradation It was suspected that previous difficulty in obtaining a reproducible protective affect using RNA might be due to degradation by RNAase both during extraction and after injection into the mice The RNAase activity in serum and lymph node cells was measured Polyvinyl sulphate was found to be an effective RNAase inhibitor and was therefore used in the RNA extraction procedures

REFERENCES

1 *Alexander P Delorme E F Hamilton L D G & Hall J G* Effect of nucleic acids from immune lymphocytes on rat sarcomata Nature 213 569–72 1967

2 *Bloom B R & Chase M W* Transfer of delayed type hypersensitivity A critical review and experimental study in the guinea pig Progr Allergy *10* 151–255 1967

3 *Cater D B & Waldmann H* The effect on the growth of BP8 ascites tumour in C3H C57 or C3H mice of lymphocyte preparations from C57 mice injected with BP8 cells and Freund s adjuvant Brit J Cancer *21* 124–36 1967

4 *Dutton R W* *In vitro* studies of immunological responses of lymphoid cells. Adv Immunol *6* 253–336 1967

5 *Glick J L & Goldberg A R* The action of thymus DNA on L1210 leukemia cells Trans N Y Acad Sci *28* 741–53 1966

6 *Gowans J L & McGregor D D* The immunological activities of lymphocytes Prog Allergy *9* 1–78 1965

7 *Krechetova G D Chudinova I A & Shapot V S* O svoistvah polivinilsulfata kak ingibitora ribo i dezoksiribonukleaz Biokhimiia 28 682–93 1963

8 *Rook C A W & Cater D B* The effect of lymph node cells from specifically sensitised mice on the growth of BP8 ascites tumour in vivo and "in vitro Brit J exper Path 49 241–50 1968

9 *Roth J S* Some observations on the assay and properties of ribonucleases in normal and tumour tissues Methods in Cancer Research 3 1 3–242 1967

10 *Scherrer K & Darnell J E* Sedimentation characteristics of rapidly labelled RNA from Hela cells Biochem Biophys Res Comm 7 486–90 1962

11 *Wilson D B & Billingham R E* Lymphocytes and transplantation immunity Adv in Immunol 7 189–273 1967

Acta path microbiol scand 77 201-217 1969

The University Institute of Pathological Anatomy
(Head Professor G Teilum M D) Copenhagen Denmark

CYTOCHEMICAL STUDY OF CASEIN INDUCED AND NITROGEN MUSTARD ACCELERATED AMYLOIDOSIS IN MICE

By

JERZY KAZIMIERCZAK

Received 10 iv 69

In recent years increasing evidence has been provided indicating a cellular origin of amyloid

In a series of contributions to this topic *Teilum* (1952 1954 1956 1957 1964) elaborated the biphasic theory of amyloid formation *in which* the essential role of cells of the reticulo endothelial system has been stressed This author indicated that a proliferation of RNA synthetizing pyroninophilic cells occurs in the initial preamyloid phase while in the second phase PAS positive cells appear whose intracytoplasmic substance when liberated precipitates *in situ* in the form of amyloid Acording to *Christensen* (1960) the cells responsible for amyloid formation were considered to be metalophilic and *Ranløv* (1966) demonstrated their phagocytic properties

The present histochemical study of some oxidative and hydrolytic enzymes during the course of development of casein induced amyloidosis and amyloidosis accelerated with nitrogen mustard aims to produce some more information of the functional characteristics of the cells involved in amyloid formation

MATERIAL AND METHODS

81 female C3H mice four to six weeks old weighing approximately 20 gm were used throughout the experiments They were fed on oatmeal and had free access to water

Experiment A

30 mice were given subcutaneous injections of casein while 15 mice were used as controls

0 5 ml of a 5 per cent solution of casein in 0 25 per cent NaOH (final pH 10 1) prepared according to the method adapted by *Christensen* (1963) was injected 5

This investigation was supported by The Danish League Against Rheumatism

Requests for reprints should be addressed to Jerzy Kazimierczak, The University Institute of Pathological Anatomy 2100 Copenhagen Ø Frederik V s Vej 11 Denmark

days a week in different sites of the back Two injected and one control mice were sacrificed by cervical dislocation at 2 to 6 day intervals up to the 42nd day following the initial injection of casein

Small pieces of spleens livers kidneys and peripheral (axillary) lymph nodes obtained immediately after death of these three animals were placed on thin cork plates prepared separately for each organ (the control organ was always placed between the two from the casein treated mice) The material was rapidly frozen in isopentane chilled with solid carbon dioxide and mounted in a Pearse Slee cryostat at —20 C

The remaining parts of the organs were fixed in 10 per cent neutral formalin and embedded in paraffin Paraffin sections cut at 4 µ were regularly stained with haematoxylin eosin periodic acid Schiff (PAS) stain methyl green pyronine and alkaline Congo red

Enzymatic histochemistry was performed on the fresh frozen cryostat serial sections usually on the day of death of the animal

Oxidative enzymes succinate dehydrogenase (succinate DH) diphosphopyridine diaphorase (DPNH diaphorase) glucose 6 phosphate dehydrogenase (glucose 6 phosphate DH) and *lactate dehydrogenase (lactate DH)* were demonstrated in unfixed frozen sections by the methods recommended by *Barka & Anderson* (1963) using Nitro blue tetrazolium as hydrogen acceptor Sodium azide as a respiratory inhibitor (*Pearse 1960*) was added to the medium for demonstration of glucose 6 phosphate DH

Hydrolytic enzymes were demonstrated in sections air dried for approximately one hour *Nonspecific esterase (esterase)* was demonstrated in unfixed sections using hexazonium pararosanilin as azo dye and alpha naphtyl acetate as the substrate (*Barka & Anderson* 1963) The method of *Wachstein & Meisel* (1957) was used to demonstrate *adenosine triphosphatase* (*ATPase*) and prior to incubation the sections were fixed in cold (—2 C) 6 per cent neutral formalin for 5 minutes (*Wachstein et al 1962*) The lead technique of *Gomori* with the slight modification by *Barka & Anderson* (1962) was employed to demonstrate *acid phosphatase* in sections prefixed in 10 per cent formalin for one hour at +4 C The incubation time for the demonstration of *ATPase* and *esterase* was 15 minutes at room temperature and for *acid phosphatase* one hour at 37 C

Occasionally as a control of Gomori s method for *acid phosphatase* the naphtyl phosphate hexazonium pararosanilin method of *Barka* (1960) was used Whether one or the other method was used the location of *acid phosphatase* was the same

In all histochemical reactions for enzymatic activity served as control sections incubated in media lacking the substrates or sections treated for 5 minutes with hot (90 C) water otherwise incubated in full strength media No enzymatic activity was detectable in either case

All the histochemical sections were counterstained with one per cent methyl green and mounted in glycerol gelatine

Frozen sections next to those used for enzymatic histochemistry were regularly stained with methyl green pyronine PAS stain and occasionally with alkaline Congo red and Giemsa stain

Experiment B

36 mice were used in this experiment (Table 1) 18 mice were treated with casein for three weeks following which they received one (Group I) two (Group II) or three (Group III) subcutaneous injections of 0 05 mg of nitrogen mustard (Erasol IDO®)— at 48 hours intervals in groups II and III These animals were killed consecutively the day after the last injection of nitrogen mustard together with the mice treated with casein only during the same period (Group IV

Nine mice were treated with nitrogen mustard alone (Group V) corresponding to those in groups I II and III and sacrificed at the same time

All organs were examined in paraffin sections stained with haematoxylin eosin methyl green pyronine alkaline Congo red and PAS stain Additionally histochemical examination of *acid phosphatase* and staining with methyl green pyronine PAS reagent and Prussian blue were performed on fresh frozen serial sections identical to the procedure described in Experiment A

RESULTS

Each examined section contained identical organs derived both from test and control animals sacrificed simultaneously This arrangement of the material permitted to a certain degree an objective evaluation of the changes to occur in the tissues during the experiments

The cycle of casein induced amyloidosis is represented by two phases the initial *preamyloid phase* and the second *amyloid phase* The appearance of small amyloid desposits within a tissue is regarded as the turning point between the preamyloid phase and the amyloid one The effect of nitrogen mustard injections will be described separately

The Preamyloid Phase

Already during the first week of casein treatment a distinct enlargement of the spleen and lymph nodes was observed At this stage also a general decrease in staining intensity in the enzymatic reactions was present in all the organs Besides in the subcapsular region of the spleen a permanent decrease in the number of cells containing *adenosine triphosphatase* (Fig 1) and *acid phosphatase* (Fig 2) was observed

Following these initial changes in all the organs of casein treated mice an increase in number of cells staning strongly with methyl green pyronine took place In the spleen the pyroninophilic cells were aggregated in the vicinity of the blood vessels and trabeculae or dispersed throughout the red pulp The dispersed cells were usually much larger than those in the aggregates and they contained a vesicular mucleus with little peripheral chromatin and a prominent strongly pyroninophilic nucleolus In the final stage of the first phase the large pyroninophilic cells dominated the red pulp They were regularly found at the border of or mixed with the *aggregated cells* suggesting a common origin of these *seemingly two types* of cells (Fig 5) In the enzymatic staining reactions all the pyroninophilic cells exhibited activity of *ATPase succinate DH,* and *glucose 6 phosphate DH* In the reaction for *glucose 6 phosphate DH* (Figs 11 and 12) the large pyroninophilic cells however appeared usually to be more intensively stained than the small pyroninophils while the latter exhibited stronger staning than the former cells following the reaction for *ATPase* (Fig 13) and *succinate DH*

In the other enzymatic staining reactions the perifollicular *zones of* the spleens of the casein treated animals contained increased numbers of cells strongly stained in the reactions for *esterase* (Fig 3) *lactate DH DPNH diaphorase* and *acid phosphatase* (Fig 2) as compared with spleens of the control animals The cells containing *acid phosphatase* appeared to be moderately *stained* also in the reaction for glucose 6 phosphate DH (Fig 12) The cells appeared in the perifollicular zone and in addition they were found spread throughout the

splenic tissue including the follicles These cells were relatively small with ragged outlines and frequent cytoplasmic extensions At the end of the pre amyloid phase some cells containing *acid phosphatase* assumed a somewhat more regular shape

The increase in number of cells containing *acid phosphatase* during the pre amyloid phase was also evident in other organs A most significant increase was observed in the liver where Kupffer cells appeared to be particularly numerous in the reaction for the latter hydrolase (Fig 4)

During the pre amyloid phase an increase in number of giant cells and polymorphonuclears was observed in the spleen Many of the latter were acidophilic granulocytes and they appeared to be very active in the reaction for *glucose 6 phosphate DH* Besides in some preparations cells with large strongly PAS positive inclusions were occasionally found They resembled much Foa Kurloff cells

The Second Amyloid Phase

About 22 days after initiation of the casein injections the first amyloid deposits appeared in the spleen This substance was primarily found in the form of small deposits of a PAS positive material placed in the perifollicular zone close to the sinusoids In frozen sections and in paraffin sections stained with alkaline Congo red the amyloid assumed a bright red colour and in polarized light it showed a vivid green birefringence

In the liver and the kidney the amyloid appeared a few days later than in the spleen and in these organs the substance was observed to develop in different sites at different times In the liver it developed first adjacent to the hepatic interlobular veins and later close to the Kupffer cells while in the kidney (about three days later than in the

---

Figs 1–4

Figs 1–3 Each figure shows spleen of mouse treated with casein for 10 days (upper) and spleen of untreated control mouse (lower) cut and incubated together with a view to demonstrating enzymatic activity

Fig 1 *Adenosine triphosphatase* The most intense staining is seen in the cells of red pulp of the normal spleen (lower) Note the disappearance of activity in the cells of subcapsular region and appearance of active cells in centre of one follicle (upper middle) of casein treated mouse (× 40)

Fig 2 *Acid phosphatase* Serial section adjacent to that shown in Fig 1 Increase in number of active cells around the follicle of casein treated mouse with relatively small number of active cells in subcapsular region (× 40)

Fig 3 *Nonspecific esterase* Increase in number of strongly stained cells around the follicles and moderately stained cells in red pulp in the casein treated mouse is seen (× 40)

Fig 4 *Acid phosphatase* in liver The number of reactive Kupffer cells in mouse treated for 20 days with casein (upper) is seen to be increased as compared to findings in the liver of control mouse (lower) (× 40)


1
2
3
4

liver) it developed first in the interstitium and then in the centrilobular regions of the glomeruli

After 26 days of casein treatment a small quantity of amyloid was also found in the peripheral (axillary) lymph nodes close to the blood vessels Although the areas occupied by amyloid in spleen liver, and kidney successively increased as the injections of casein were continued the amount of this substance in the peripheral lymph nodes was never significant even in the animals killed at the end of the experiment

The appearance of amyloid within the perifollicular zones of the spleen was at the same sites followed by a rapid decrease in the number of cells which—during the pre amyloid phase—were observed to be strongly stained in the reactions for *lactate DH DPNH diaphorase esterase*, and *acid phosphatase* The number of the pyroninophilic cells around the perifollicular zone also became smaller although the pyroninophilic cells in the rest of the red pulp were very abundant and exhibited almost the same pattern of enzymatic activities as in the pre amyloid phase (Fig 14) In the frozen serial sections stained with methyl green pyronine alternately with the reaction for *acid phosphatase* several cells somewhat similar to the pyroninophilic reticular cells did not stain with methyl green pyronine but exhibited a prominent activity of *acid phosphatase* These cells were very characteristic of the second amyloid phase They were usually situated close to the amyloid deposits (Fig 15) and differed from the cells which in the first phase were seen to contain *acid phosphatase* in that their sizes were larger and outlines more regular However small unregularly shaped cells with strong *acid phosphatase* activity were also present intermingled with the large cells at the border of the amyloid substance Only if amyloid developing around one follicle coalesced with that developing around the neighbouring one (amyloid degree

---

Figs 5–10

Fig 5 Spleen of mouse injected for 18 days with casein Methyl green pyronine stain Pyroninophilic cells of various sizes some with strongly pyroninophilic nucleoli are seen (× 1000)

Fig 6 Spleen of mouse treated for four weeks with casein Characteristic cells with cytoplasm well stained in PAS reagent are seen in area of *developing* PAS positive amyloid Periodic acid Schiff stain (× 400)

Fig 7 Spleen of mouse treated with casein for three weeks followed by two injections of nitrogen mustard *Acid phosphatase* activity in large reticular cells Note strong activity of *acid phosphatase* in other smaller cell (× 1000)

Fig 8 Spleen of mouse treated as that shown in Fig 7 Many cells with intracytoplasmatic inclusions are mixed with pyroninophilic cells Methyl green pyronine stain (× 1000)

Fig 9 Spleen of mouse casein treated for three weeks followed by two *injections* of nitrogen mustard Methyl green pyronine stain (× 100)

Fig 10 Serial section adjacent to that shown in Fig 9 demonstrates *acid phosphatase* activity at sites where pyroninophilic cells (Fig 9) are not present (× 100)

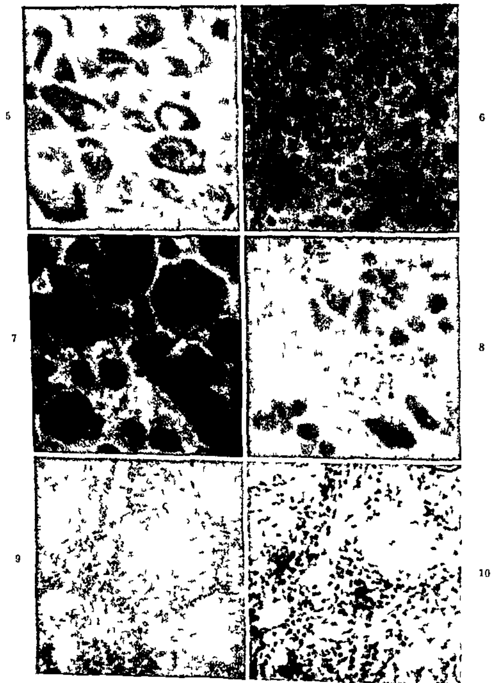

5
6
7
8
9
10

TABLE 1

The Extent of Amyloid Formation in Various Groups of Mice Treated with Casein and Nitrogen Mustard

Group	Treatment	Number of mice developing amyloidosis/ total	Mean degree of amyloidosis (*Christensen & Hjort* 1959) in the mice with developed amyloid
I	Casein 21 days + one injection of nitrogen mustard	2/6	++
II	Casein 21 days + two injections of nitrogen mustard	3/6	+++
III	Casein 21 days + three injections of nitrogen mustard	4/6	++++
IV	Casein 21 days	4/9	++
V	One to three injections of nitrogen mustard	0/9	-

great deal of *acid phosphatase* positive cells contained in their cyto plasm numerous inclusions which in a preparation stained with Prussian blue appeared to contain iron (Fig. 19) These cells were intermingled with the remaining pyroninophilic cells (Fig 20) being similar to these by the shape and size of their nuclei (Fig 8) Since no other cells in these areas showed such similarity there seems little doubt that the decrease in number of the pyroninophilic cells was at

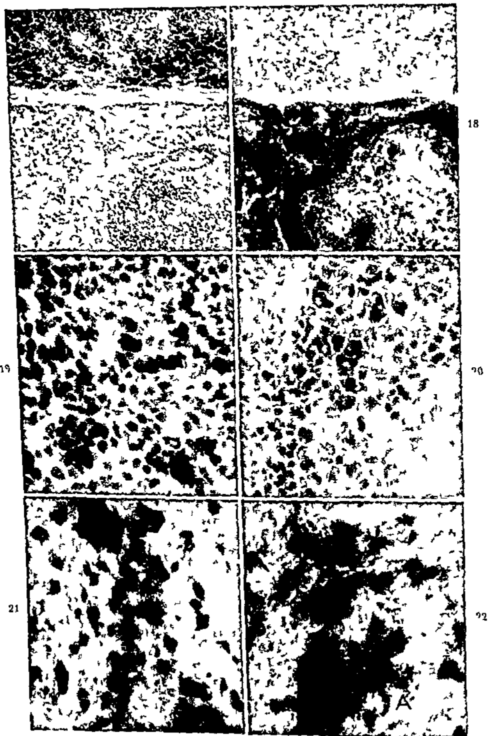
Figs 17–22

Figs 17 and 18 Spleen of mouse treated with casein for three weeks (upper) together with spleen of mouse treated with the same number of casein injections followed by two injections of nitrogen mustard (lower) No amyloid was yet present in these spleens Distinct decrease of pyroninophilia (Fig 17) and increase in number of cells containing *acid phosphatase* (Fig 18) *in the nitrogen mustard treated mouse is demonstrated* Fig 17—methyl green pyronine stain Fig 18—Gomori's acid phosphatase reaction (× 400)

Fig 19 *Spleen of mouse* treated with casein for three weeks followed by two injections of nitrogen mustard without development *of amyloid* *Several cells* of red pulp are intensively stained with Prussian blue Nuclei are counter stained with carmalum (× 400)

Fig 20 Spleen of mouse treated as that shown in Fig 19 *Several pyroninophilic* cells and cells containing inclusions are demonstrated *Methyl green pyro-*nine stain (× 400)

Figs 21 and 22 *Acid phosphatase* activity at the border of perifollicular zone in spleen of mice treated as that shown in Fig 19 without amyloid (*Fig 21*) and with amyloid already developed (Fig 22) Note the diffuse *reaction in* latter (× 400)



17
18
19
20
21
22

least in part due to a transformation of the latter to another functional form In addition several other large and regulary shaped cells much like the large pyroninophilic reticular cells showed *acid phosphatase* activity (Fig 7) although no iron substance was detectable in their cytoplasm

In the animals developing amyloid the numbers of *acid phosphatase* containing cells and of pyroninophilic cells were much smaller than those in animals without amyloidosis Still however the reaction for *acid phosphatase* in the red pulp exhibited a complementary negative picture of the staining with methyl green pyronine the pyroninophilic cells were present only at sites where the cells containing *acid phosphatase* were absent (Figs 9 and 10) Many cells containing *acid phosphatase* appeared also to be stained in the PAS reagent

In the animals in groups I, II and III which failed to develop amyloid *acid phosphatase* was localized entirely intracytoplasmatically (Fig 21) On the other hand in the animals in the same groups with developed amyloidosis this hydrolase was also present within masses of apparently new amyloid deposits at the border of the perifollicular zone in coalescence with an unreactive substance which presumably represented the earlier developed amyloid (Fig 22)

The presence of iron containing cells and the increase in number of cells with *acid phosphatase* activity were also observed in the animals injected with nitrogen mustard alone (Group V) None of these animals developed amyloid and three mice which received three injections of nitrogen mustard died spontaneously within 24 hours following the last injection

DISCUSSION

In the present study the patterns of some oxidative and hydrolytic enzymes are described together with the histological changes occuring in the organs of female C3H mice during the development of casein induced amyloidosis In addition *acid phosphatase* activity was histochemically evaluated in mice treated for 21 days with casein followed by injection with nitrogen mustard Since the morphological alterations observed in these experiments were most significant in the spleen this organ was primarily considered

The sequence of time of development of amyloid and its location in the organs observed in the present study are almost identical to findings in C3H mice reported by *Christensen* (1963) and *Ranlov & Christensen* (1968) and are the features usually observed in our laboratory The presence of amyloid in the peripheral lymph nodes has not however as yet been reported

The process of amyloid development has by *Teilum* (1957) been indicated to occur in two phases of cellular activity characterized by proliferation of pyroninophilic cells during the first phase and ap

pearance of PAS positive cells and amyloid in the second This biphasic development of amyloid is confirmed in the present experiments In the enzymatic staining reactions it was possible to sort out some changes in enzymatic activities related to such phases

The initial general diminishment and the almost complete disappearance of enzymatic activity in some cells were presumably the expected disturbances to occur in antigen treated animals as a result of a remodelling of the cell population (*Congdon* 1964) protoplasmic excitation (*Dannenberg et al* 1963) or saturation of macrophages (*Barla et al* 1961)

Following these initial alterations a proliferation of pyroninophilic reticular cells well supplied in *ATPase, succinate DH* and *glucose 6 phosphate DH*, took place While *succinate DH* in these cells obviously indicated energy supplying Krebs cycle the presence of *glucose 6 phosphate DH* (in association with pyroninophilia) apparently demonstrated a provision of pentose sugar for the synthesis of nucleotides (*Pearse* 1958 1960)

The development of amyloid coincided with a decrease in the number of pyroninophilic cells and the appearance at corresponding places of large cells containing *acid phosphatase* In turn the location of the latter corresponded to the cells well stained with the PAS reagent and originally described by *Teilum* (1956) as the glycoprotein producing cells of the reticulo endothelial system directly responsible for amyloid formation *in situ* In the present study these cells are believed to be the earlier pyroninophilic reticular cells which in the final stage of their acticity became filled up with selfdestructive cytolysomes (*Novikoff* 1960) similarly to other degenerating cells observed in various necrotizing tissues (*Novikoff* 1959 *Becker & Barron* 1961) The presence of lysosome or cytolysome like bodies in the cells apparently responsible for amyloid deposition was also demonstrated by *Ranlov & Wanstrup* (1967) in ultrastructural investigations according to which degenerating processes occured in these cells The lysosomes in addition to *acid phosphatase* contain several other hydrolytic enzymes (*Du Duve* 1959) which are capable of destroying most of the important cell constituents In the present study however it cannot be proved whether or not the action of the lysosomal hydrolases whithin the hyperimmunized cell is amyloidogenic On the other hand it was observed that extracellularly deposited amyloid contained also some *acid phosphatase* suggesting that the hydrolase (or hydrolases) when released from the lysosomes into the soluble portion of the cell destroyed its structure thereby liberating the cellular product which then precipitated *in situ* as amyloid

The cells stimulated during the casein induced amyloidosis appeared to differentiate in a manner similar to that of other cells of the reticulo endothelial system observed *in vitro* (*Cohn & Wiener* 1963 a b *Dannenberg et al* 1963 *Quaglino & Hayhoe* 1965) where

least in part due to a transformation of the latter to another functional form In addition several other large and regularly shaped cells much like the large pyroninophilic reticular cells showed *acid phosphatase* activity (Fig 7) although no iron substance was detectable in their cytoplasm

In the animals developing amyloid the numbers of *acid phosphatase* containing cells and of pyroninophilic cells were much smaller than those in animals without amyloidosis Still however the reaction for *acid phosphatase* in the red pulp exhibited a complementary negative picture of the staining with methyl green pyronine the pyroninophilic cells were present only at sites where the cells containing *acid phosphatase* were absent (Figs 9 and 10) Many cells containing *acid phosphatase* appeared also to be stained in the PAS reagent

In the animals in groups I II and III which failed to develop amyloid *acid phosphatase* was localized entirely intracytoplasmatically (Fig 21) On the other hand in the animals in the same groups with developed amyloidosis this hydrolase was also present within masses of apparently new amyloid deposits at the border of the perifollicular zone in coalescence with an unreactive substance which presumably represented the earlier developed amyloid (Fig 22)

The presence of iron containing cells and the increase in number of cells with *acid phosphatase* activity were also observed in the animals injected with nitrogen mustard alone (Group V) None of these animals developed amyloid and three mice which received three injections of nitrogen mustard died spontaneously within 24 hours following the last injection

DISCUSSION

In the present study the patterns of some oxidative and hydrolytic enzymes are described together with the histological changes occuring in the organs of female C3H mice during the development of casein induced amyloidosis In addition *acid phosphatase* activity was histochemically evaluated in mice treated for 21 days with casein followed by injection with nitrogen mustard Since the morphological alterations observed in these experiments were most significant in the spleen this organ was primarily considered

The sequence of time of development of amyloid and its location in the organs observed in the present study are almost identical to findings in C3H mice reported by *Christensen* (1963) and *Ranløv & Christensen* (1968) and are the features usually observed in our laboratory The presence of amyloid in the peripheral lymph nodes has not however as yet been reported

The process of amyloid development has by *Teilum* (1957) been indicated to occur in two phases of cellular activity characterized by proliferation of pyroninophilic cells during the first phase and ap

with nitrogen mustard only no such effect can be expected since cells which passed the pyroninophilic phase (*Teilum* 1964) are not present at the sites where the process of amyloid formation could be initiated

SUMMARY

The activity of oxidative and hydrolytic enzymes was studied histochemically in the organs of mice during the course of casein induced amyloidosis Also the effect of nitrogen mustard on casein treated mice was investigated using the reaction for acid phosphatase Subsequently serial sections were stained with methyl green pyronine and PAS stain The increase in pyroninophilia in the preamyloid phase was found to be followed by a general increase in the oxidative enzymes activity of which the activity of *glucose 6 phosphate dehydrogenase* appeared to be most distinct in the pyroninophilic cells where the synthesis of RNA presumably occurred

An increase in the number of cells with hydrolytic enzymes was also observed and the cells containing *acid phosphatase* appeared to be most intimately related to the process of amyloid formation In the second amyloid phase the cells containing *acid phosphatase* were always present at the sites of developing amyloid and their position and size were often found to be identical with those of the cells stained with PAS The cells with abundant *acid phosphatase* activity were particularly numerous in the mice treated with casein and nitrogen mustard and were appearing successively at the sites where a decrease of the pyroninophilic cells was observed

A possible transformation of the pyroninophilic cells into cells with abundant *acid phosphatase* and their eventual relation to amyloid formation *in situ* is discussed

REFERENCES

Barka T A simple azo dye method for histochemical demonstration of acid phosphatase Nature (Lond) *187* 248–249 1960

Barka T Schaffner F & Popper H Acid phosphatase and reticuloendothelial system Lab Invest *10* 590–607 1961

Barka T & Anderson P J Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler J Histochem Cytochem *10* 741–753 1962

Barka T & Anderson P J Histochemistry Theory Practice Bibliography Hoeber Medical Division Harper & Row Publishers New York 1963

Becker N H & Barron K D The cytochemistry of anoxic and anoxic—ischemic encephalopathy in rats I Alterations in neuronal lysosomes identified by acid phosphatase activity Amer J Path *38* 161–175 1961

Christensen H E Metalophil reticular cells in experimental mouse amyloidosis Acta path microbiol scand *50* 29–41 1960

Christensen H E Amyloidosestudier Thesis Copenhagen 1963

Christensen H E & Hjort G H X irradiation as accelerating factor in caseinate induced amyloidosis in mice Acta path microbiol scand *47* 140–152 1959

Cohn Z A & Benson B The differentiation of mononuclear phagocytes Morphology cytochemistry and biochemistry J exp Med *121* 153–170 1965

Cohn Z A & Wiener F The particulate hydrolases of macrophages I Comparative enzymology isolation and properties J exp Med *118* 991–1008 1963 a

Cohn Z A & Wiener F The particulate hydrolases of macrophages II Biochemical and morphological response to particle ingestion J exp Med *118* 1009–1020 1963 b

Congdon C C The early histologic effects of antigenic stimulation Arch Path *78* 83–96 1964

Dannenberg A M Jr Walter P C & Kapral F A A histochemical study of phagocytic and enzymatic functions of rabbit mononuclear and polymorphonuclear exudate cells and alveolar macrophages II The effect of particle ingestion on enzyme activity two phases of *in vitro* activation J Immunol *90* 448–465 1963

Du Duve C The function of intracellular hydrolases Exp Cell Res Supp *7* 169–182 1959

Fishman M Antibody formation *in vitro* J exp Med *114* 837–856 1961

Fishman M & Adler F L *Antibody formation initiated in vitro II* Antibody synthesis in X irradiated recipients of diffusion chambers containing nucleic acid derived from macrophages incubated with antigen J exp Med *117* 595–602 *1963*

Frei P C Benacerraf B & Thorbecke G J Phagocytosis of the antigen a crucial step in the induction of the primary response Proc Nat Acad Sci USA *53* 20–23 196

Howard J G Activation of the reticulo endothelial cells of the mouse liver by bacterial lipopolysaccharide J Path Bact *78* 465–470 1959

Lapresle C & Webb T Degradation of a protein antigen by intracellular enzymes In Cellular aspects of immunity *Wolstenholme C E W & O Connor M* (eds) *Ciba Foundation Symposium Little Brown & Co Boston 1960 pp 44–57*

Malec J Kornacka L Wojnarowska M & Zalrewski K The activation of nuclear nucleoproteins during phagocytosis Exp Cell Res 25 457–460 1961

Novikoff A B The proximal tubule cell in experimental hydronephrosis J Biophys & Biochem Cytol *6* 136–138 1959

Novikoff A B Biochemical and staining reactions of cytoplasmic constituents In Developing cell systems and their control *Rudnick D* (ed) Ronald Press Co New York 1960 pp 167–203

Pearse A G E *Extension of limits of cellular pathology the role of enzyme histo*chemistry J clin Path (Lond) *11* 520 534 1958

Pearse A G E Histochemistry theoretical and applied Churchill Ltd (2nd Ed) London 1960

Quaglino D & Hayhoe F G J Metabolic changes in short term *in vitro* cultures of normal and leukemic cells In Current research in leukemia *Hayhoe F G J* (ed) Cambridge Univ Press London 1965

Ranlov P Phagocytosis in experimental mouse amyloidosis Acta path microbiol scand *68* 19–28 1966

Ranlov P Immunology and pathogenesis of experimental amyloidosis Dan med Bull *15* 289–296 1968

Ranlov P & Christensen H E The *inversion stage* of experimental mouse amyloidosis determined by the accelerating effect of nitrogen mustard after various lengths of pretreatment with casein Acta path microbiol scand *72* 233–236 1968

Ranlov P & Wanstrup J Ultrastructural investigations on the cellular morphogenesis of experimental mouse amyloidosis Acta path microbiol scand *71* 575–591 1967

Teilum G Cortisone ascorbic acid interaction and the pathogenesis of amyloidosis Mechanism of action of cortisone on mesenchymal tissue Ann rheum Dis *11* 119–136 1952

Teilum G Studies on pathogenesis of amyloidosis effect of nitrogen mustard in *inducing amyloidosis* J Lab clin Med *43* 367–374 1954

Teilum G Periodic acid Schiff positive reticulo endothelial cells producing glycoproteins functional significance during formation of amyloid Amer J Path *32* 945–959 *1956*

Teilum G Pathogenesis of amyloidosis in the light of recent cytochemical investigations Acta rheum scand *3* 164–168 1957

Teilum G Pathogenesis of amyloidosis The two phase cellular theory of local secretion Acta path microbiol scand 61 21–45 1964

Thorbecke G J Old L J Benacerraf B & Clarke D A A histochemical study of acid and alkaline phosphatase in mouse livers during various conditions modifying activity of the reticuloendothelial system J Histochem Cytochem 9 392–399 1961

Wachstein M & Meisel E Histochemistry of hepatic phosphatases at a physiologic pH Amer J clin Path 27 13–23 1957

Wachstein M Bradshaw M & Ortiz J M Histochemical demonstration of mitochondrial adenosine triphosphatase activity in tissue sections J Histochem Cytochem 10 65–74 1962

Weiss L P & Fawcett D W Cytochemical observations on chicken monocytes macrophages and giant cells in tissue culture J Histochem Cytochem 1 47–65 1953

Wiener J Spiro D & Zunker H O A cellular study of tuberculin sensitivity Amer J Path 47 723–763 1965

Acta path microbiol scand 77 218-22? 1969

Department of Veterinary Virology and Immunology The Royal Veterinary and Agricultural University Copenhagen Denmark.

IMMUNITY TO *ASCARIS SUUM*

I *Immunity Induced in Mice by Means of Material from Adult Worms*

By

ERLING BINDSETH

Received 27 iii 69

Much immunological work has been done on *Ascaris suum*, but the results have not thrown much light upon the question why the immunity is closely associated with the actual infection i e the presence of the living parasites in the host and why somatic antigens generally have proved to be incapable of stimulating immunity to parasites As far as *Ascaris suum* is concerned *Soulsby* (1963) has shown that it is possible to immunize guinea pigs against the larval migration by means of somatic antigens from larvae recovered from lungs of rabbits infected artificially with *Ascaris suum* Furthermore excretory and secretory products from second and third stage larvae are effective in stimulating an immunity to *Ascaris suum* as shown by *Crandall & Arean* (1965) and *Soulsby* (1957) respectively Experiments by *Martin* (1926) and *Sprent & Chen* (1949) showed no effectiveness of adult *Ascaris* material as regards the capacity of stimulating immunity Contrary to that are suggestions by *Wagner* (1933) *Kerr* (1938) *Fallis* (1948) *Berger & Wood* (1964) and *Lejkina* (1965)

Prior to the institution of investigations of the aspects of immunity to *Ascaris suum* the following preliminary study was devised to see how different immunization procedures might stimulate immunity (resistance) to *Ascaris suum* infections in mice using an extract of adult worms

The finding of a number of larvae in the lungs of immunized mice lower than the number in non immunized mice served as the criterion of immunity

The author is greatly indebted to Dr *E Andresen* and Mr *K Christensen* Agronomist Department of Animal Genetics The Royal Veterinary and Agricultural University Copenhagen for help and guidance with the statistical analysis

The work was carried out with financial support from the *Danish State Research Foundation* (*Statens almindelige Videnskabsfond*)

MATERIALS AND METHODS

Mice Male white mice (N M R I) were purchased at a breeding centre According to *Casarosa* (1967) *Ascaris suum* infections in mice older than 6–7 weeks are likely to give very variable results as regards the degree of larval migration Hence it was preferred to use mice that were only 2 weeks old at the beginning of the experiments

Antigen for immunization Adult male and female *Ascaris suum* were obtained from a local abattoir At the laboratory the worms were washed repeatedly with tap water and saline Equal amounts of male and female worms and saline were mixed for 20 min in a homogenizer (Servall Omnimixer 14 000 r p m) The whole portion was placed in the refrigerator overnight under magnetic stirring After centrifugation (20 min at 3 000 r p m (c 1 400 g) the supernatant was stored at about —23 C and used as antigen Very little is known to day about the nature of the antigenic function of *Ascaris suum* in immunity so in order to calculate the strength of the antigen a possible function in immunity was not ascribed to any particular component in the supernatant but a determination of dry matter percentage was preferred It was carried out according to *Jepsen* (1944) and was found to be 7 5

Infective eggs for infection Adult female worms were obtained from a local abattoir The distal parts of the uteri (approximately 2 cm) were removed and suspended in 20 ml of saline After mixing at low speed in the homogenizer for 10 min the eggs were isolated The suspension of eggs was mixed with an equal volume of NaOCl (c 5 per cent active chlorine) and after 20 min the eggs were decoated Subsequently the eggs were washed several times in saline by centrifugation at 700–1 000 r p m (Christ Simplex) for 1–2 min The eggs were then resuspended in 0 1 n H_2SO_4 and placed in *Petri* dishes and incubated for 30 days at 30 C and at 20 C for 21 days During incubation the eggs were aerated by gentle agitation for some minutes daily After the incubation the eggs were tested for infectivity by oral inoculations into mice

Recovery of larvae At necropsy the lungs of the mice were removed and chopped with a knife The material was divided into 2 equal parts Live larvae were obtained from the one part by *Baermann* technique (BM) and the second part was digested in artificial gastric fluid (GF) for recovery of the total number of larvae i e larvae retained in the tissue *in vivo* and larvae killed by the treatment The *Baermann* apparatus was incubated at 37 C for 4 hrs and the dishes with tissue to be digested were incubated overnight at 37 C One thousand ml of artificial gastric fluid consisted of 10 ml of HCl (conc) 2 g of NaCl and 0 6 g of Pepsin 1 10 000 NF according to *Casarosa* (1967)

Larval counts The larvae recovered from the lungs by the preceding techniques were counted in *Petri* dishes under the microscope To calculate if any difference in mean larval counts was statistically significant, an F test and a t test were carried out the former being applied to determine whether it was reasonable to use a t test A null hypothesis was considered in both groups of experiments

EXPERIMENTS

Experiments 1 2 and 3 Each experiment included 20 mice and each mouse received 0 5 ml of the *Ascaris* extract intraperitoneally every second day doses totalling 2 0 ml In experiments 1 2 and 3 respectively the mice were challenged with 3 000 infective eggs by stomach intubation 7 18 and 23 days later In each experiment 20 non injected mice served as controls and were challenged at the same time and with the same dose as the immunized mice All mice were killed 7 days after challenge

Experiments 4 5 and 6 There were 20 mice in each experiment and each mouse received 0 5 ml of the *Ascaris* extract intraperitoneally every second day doses totalling 2 0 ml Two weeks later the mice were re injected with 0 5 ml each Seven days later the mice were challenged with 3 000 eggs In each experiment 20 non injected mice served as controls and were challenged with 3 000 eggs at the same time as the re immunized mice All animals were sacrificed 7 days after challenge

Deaths mainly occurring in relation to the first injections and to the application

by stomach tube are responsible for the differences in numbers of animals stated above and in Tables 1 and 2

RESULTS

The mean number of larvae recovered from the lungs are seen in Tables 1 and 2 It will appear from Table 1 that there was no difference

TABLE 1

Mean Number of Larvae Recovered from the Lungs of Mice Immunized with Adult Ascaris suum Extract and not Subjected to Re Immunization after a Challenge with Infective Eggs

Exper	Treatment	TC	No animals	Mean No larvae (½ lung)	
				BM	GP
1	Immunized intraperitoneally	7	16	42	49
	Infected controls	7	20	56	53
2	Immunized intraperitoneally	18	13	100	110
	Infected controls	18	10	67	84
3	Immunized intraperitoneally	23	13	57	61
	Infected controls	23	10	51	51

Abbreviations BM = *Baermann* technique GF = Digestion in artificial gastric fluid TC = Time of challenge Days after last injection

TABLE 2

Mean Number of Larvae Recovered from the Lungs of Mice Re Immunized with Adult Ascaris suum *Extract after a Challenge with Infective Eggs*

Exper	Treatment	TC	No animals	Mean No larvae (½ lung) and statistics					
				BM ($\bar{x}$)	F test value	t test value	GF ($\bar{x}$)	F test value	t test value
4	Re immunized intraperitoneally	7	13	29			3[illegible]		
	Infected controls	7	14	66	2 4	3 3 (2, DF)	65	2 9	[illegible] 4
5	Re immunized intraperitoneally	7	14	43		–	48		–
	Infected controls	7	12	96	6 [illegible]†		96	7 1†	
6	Re immunized intraperitoneally	7	11	146			144		
	Infected controls	7	19	202	1 3	1 1 (90 % probability 26 DF)	215	1 8	1 3 (90 % probability 26 DF)

Abbreviations BM = *Baermann* technique GF = digestion in artificial gastric fluid TC = time of challenge *Days after* last injection DF = degrees of freedom

† The variances statistically significant t test not useful (for details see the text)

Significance of t tests 0 005 > P > 0 001 0 05 > P > 0 01 P > 0 1

between the larval counts in infected controls and in mice subjected to only 4 preliminary injections and it was confirmed by the statistical analysis ($P > 0.05$)

As appears from Table 2 the results of Experiment 4 rejected the null hypothesis The differences between the mean larval counts in re immunized mice and in controls are statistically significant

Experiments 5 and 6 were devised to test this rejection Even if the F values in Experiment 5 were too high to make t tests reasonable and even if the statistical analysis of the mean larval counts in Experiment 6 showed no statistical significance there seems to be a tendency in both experiments towards a stimulation of protective immunity as the number of larvae recovered from the infected controls was higher than numbers of larvae recovered from the mice subjected to re immunization

DISCUSSION AND CONCLUSION

Until 1963 when Soulsby stimulated immunity to *Ascaris suum* infections in guinea pigs by injection of somatic material from dead third stage larvae nothing seemed to indicate convincingly that dead *Ascaris* material could give protective immunity Soulsby suggested to revise the opinion that somatic antigens were ineffective as a means by which to stimulate immunity to parasites

The main purpose of the present study was to carry out a preliminary investigation concerning the capacity of material from adult worms to stimulate protective immunity

It is evident from the figures presented in Table 1 that it was not possible with the stated immunization procedures and the chosen criterion of immunity to make mice resistant to a challenge infection whether it took place 7 18 or 23 days after the last injection

However when the preliminary series of injections is followed by a re injection 2 weeks later some effect is observed It is true that only one experiment shows statistical significance (no 4) but as findings after either of the 2 repeated injections (nos 5 and 6) clearly suggested that the number of larvae in the re injected mice was lower than *that in the controls* it is very likely that the re injection procedures have provoked some effect against the challenge infection—an effect which is worth further investigation

Furthermore the results indicate that it may not be necessary to use larval antigens to protect from the larval migration but that antigens from adult worms may *be useful as well*

SUMMARY

In a preliminary investigation the capacity of a saline *extract of adult* male and female *Ascaris suum* to stimulate immunity was carried out in mice Three groups of mice were subjected to 4 intraperitoneal in

by stomach tube are responsible for the differences in numbers of animals stated above and in Tables 1 and 2

RESULTS

The mean number of larvae recovered from the lungs are seen in Tables 1 and 2 It will appear from Table 1 that there was no difference

TABLE 1

Mean Number of Larvae Recovered from the Lungs of Mice Immunized with Adult Ascaris suum Extract and not Subjected to Re Immunization after a Challenge with Infective Eggs

Exper	Treatment	TC	No animals	Mean No larvae (½ lung)	
				BM	*GF*
1	Immunized intraperitoneally	7	16	4[illegible]	49
	Infected controls	7	20	56	53
2	Immunized intraperitoneally	18	13	100	110
	Infected controls	18	10	67	84
3	*Immunized intraperitoneally*	23	*13*	57	*61*
	Infected controls	23	10	51	51

Abbreviations BM = *Baermann* technique GI = Digestion in artificial gastric fluid TC = Time of challenge Days after last injection

TABLE 2

Mean Number of Larvae Recovered from the Lungs of Mice Re Immunized with Adult Ascaris suum Extract after a Challenge with Infective Eggs

Exper	Treatment	TC	No animals	Mean No larvae (½ lung) and statistics					
				BM ($\bar{x}$)	F test value	t test value	GF ($\bar{x}$)	F test value	t test value
4	Re immunized intraperitoneally	7	13	29			35		
	Infected controls	7	14	66	2 4	3 3 (25 DF)	6[illegible]	[illegible]9	2 4
5	Re immunized intraperitoneally	7	14	43		–	48		–
	Infected controls	7	12	96	6 [illegible]†		96	7 1†	
6	Re immunized intraperitoneally	7	11	146			144		
	Infected controls	7	19	202	1 3	1 1 (90 % probability 26 DF)	215	1 8	1 3 (90 % probability 26 DF)

Abbreviations BM = *Baermann* technique GF = digestion in artificial gastric fluid TC = time of challenge Days after last injection DF = degrees of freedom

† *The variances statistically significant t test not useful (for details see the text)*

Significance of t tests 0 005 > P > 0 001 0 05 > P > 0 01 *P > 0 1*

Acta path microbiol scand 77 2?3 234 1969

Department of Veterinary Virology and Immunology The Royal Veterinary and Agricultural University Copenhagen Denmark

IMMUNITY TO *ASCARIS SUUM*

2 *Investigations of the Fate of Larvae in Immune and Non Immune Mice*

By

ERLING BINDSEIL

Received 27 iii 69

From experiments with *Ascaris suum* infections in mice guinea pigs rabbits and pigs *Kerr* (1938) *Fallis* (1944 1948) *Sprent & Chen* (1949) *Soulsby* (1961) and *Taffs* (1968) concluded that the immune mechanism is predominantly working in the liver *Kelley & Nayak* (1964 1965) and *Maloff & Tersijski* (1967) considered the intestinal wall of importance also in pigs and mice respectively and *Wagner* (1933) working on mice considered only the gut wall of importance The conclusions were based on larval counts measurements of larvae and histological examinations

The not quite concurrent observations by the above mentioned authors make more accurate information needed

Experiments by *Brambell* (1963) and others—reviewed briefly by *Michel* (1968)—seem to indicate that larvae and adult worms of different parasites show an increasing tendency to migrate to other sites in an immune host than in a normal host The reports suggest that the habitat of the parasites deteriorates as a result of the infections thus possibly becoming less favourable for the parasites

In order to define the nature of the immunological reactions which are important from a protective point of view it is essential to know exactly in which organ or organs the parasites are arrested and destroyed

Therefore the present study was devised with a view to further enquiring into the fate of *Ascaris suum* larvae in immune and non immune mice and furthermore to investigate more thoroughly the effect of re injection procedures using adult *Ascaris* extract as a means by which to stimulate immunity—an effect recently stated by *Bindseil* (1969) The experiments include daily larval counts in liver and lung and histological examinations in mice immunized by repeated oral inoculations of *Ascaris* eggs and by re injections of adult *Ascaris* extract

The study was carried out with financial support from the *Danish State Research Foundation (Stat n almin lelige V i l nsl ab fond)*

(vaccination) The finding of a number of larvae in immune mice lower than the number in infected controls was chosen as the criterion of *immunity* (resistance)

MATERIALS AND METHODS

Mice Male white mice (N M R I) weighing 16–18 g (c 3 weeks old) were purchased at a local breeding centre Infection of the mice was carried out by oral application *of eggs by a stomach tube The mice were* slightly anaesthetized with ether during the application

Antigen for vaccination and infective eggs for infection The details of the preparation of the *Ascaris* extract and of the infective eggs as well as the details of the procedures by which the extract was re injected were the same as those described in a previous study (*Bindseil* 1969)

Histological examination At necropsy the liver the lungs and the spleen were *removed The right lobe of the liver and the mediastinal lobe of the lungs and the* spleen *in toto* were fixed in 10 per cent formalin and embedded in paraffin Sections were cut at approximately 6 μ and stained with *Harris*s haematoxylin and eosin (HE) iron haematoxylin picric acid—acid fuchsin (VG) and with methyl green pyronin (PM)

Larval counts The remaining parts of the livers and lungs were chopped with a knife and half of the material was digested in artificial gastric fluid according to *Casarosa* (1967) *The composition of the medium is cited by Bindseil* (1969)

EXPERIMENTS

The batch of mice was divided into 3 groups Two mice from each group were sacrificed and examined the day before the test infection

One group of mice was infected with 3 000 eggs and 2 mice were killed each day for 12 days after the infection

The second group of mice was subjected to a re vaccination procedure with *Ascaris* extract and the mice were challenged with 3 000 eggs 7 days after the last *injection and 2 were killed each day for 11 days after the challenge infection*

In the third and last group the mice were subjected to 3 infections at 7 days intervals with 3 000 eggs each time The mice were challenged with 3 000 eggs 13 days after the last infection and 2 mice were killed daily for 10 days after the challenge

In addition to the larval counts made on the daily samples counts were made on material from 3 mice in each group 7 and 14 days after the test infection From *these mice counts were carried out not only* on liver and lung but al o on gut wall kidney heart spleen brain and muscles of the thighs No histological examination was carried out in this experiment

RESULTS

Larval Migration

The daily number of larvae recovered is seen in Fig 1 The figures are the average of the 2 daily observations in each group Two and two *there was generally good accordance* between the counts (i g 301 and 344) but great variations occurred for example on the 1st day following the test infection in the controls 237 and 48 larvae were recovered from the livers respectively

By comparing the size of the dose of infective eggs—3 000 —with the maximum theoretical number of larvae (twice the maximal average of larvae recovered) the approximate percentage of eggs responsible for a migration of larvae was calculated By adding the averages ob

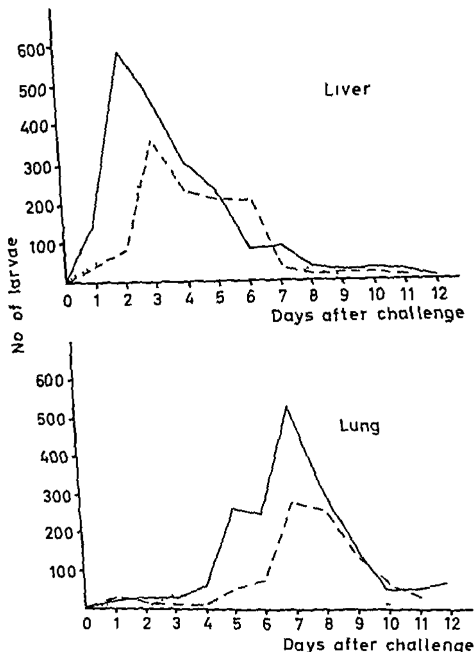


Fig 1

Distribution of larvae in mice infected orally with 3 000 infective eggs of *Ascaris suum* Average of [illegible] mice

Key ———— infected controls
- - - - - - re vaccinated with extract of adult worms infected 3 times previously with a total dose of 9 000 eggs

tained in livers and lungs separately in each group the number of larvae to be recovered after the migration to the lungs was calculated

Group 1 It appears from Fig 1 that the number of larvae in the liver after challenge is rising rapidly to a maximum number of 596 on the 2nd day Immediately after this maximum is achieved the number falls steadily coincidentally with a rise in the number of lung larvae reaching maximum—518—on the 7th day After that day lung larvae are recovered in rapidly decreasing numbers It is found that about 10 per cent of the dose have resulted in migrating larvae and approximately 83 per cent of these larvae are recovered after the migration to the lungs

Group 2 The overall pattern of migration is similar to that found in group 1 (Fig 1), except that the rise in number of liver larvae is delayed giving a maximum of 323 larvae on the 3rd day following challenge The maximum number in the lungs is 286 and is reached on the 7th day as in the controls

Much fewer larvae were recovered from these vaccinated mice Only about 21 5 per cent of the challenge dose gave rise to migrating larvae but having penetrated the gut wall once the larvae apparently behaved like larvae in control mice as about 81 per cent of the larvae were recovered from the lungs after migration through the liver

Group 3 It appears from Fig 1 that the maximum of liver larvae—269—is reached on the 2nd day after challenge Then the decrease is rapid as in the preceding groups but from now on the pattern of the migration is significantly different as the lung larvae are only recovered in very small numbers—81 in maximum So the decrease in the number of liver larvae does not coincide with a rise in the number of lung larvae

Approximately 18 per cent of the challenge dose have resulted in migrating larvae but only about 25 per cent of these larvae were recovered after migration to the lungs

The additional investigations on mice killed 7 and 14 days after challenge showed that only few larvae were observed in organs other than livers and lungs thus showing that the tendency to migration to sites other than the normal sites of predilection was not increased in immune mice (Table 1)

POST MORTEM EXAMINATION

Liver

Macroscopical appearance The gross appearance of the livers from all the groups (1–3) was rather similar However the livers from the infected controls generally seemed more mottled The colour varied from greyish brown to reddish brown

Histology The following description will apply to any of the 3 groups The lesions found in the livers from the groups were of similar

TABLF 1

Distribution of Ascaris Suum Larvae in Mice 7 and 14 Days after Challenge with 3 000 Eggs Orally Average of 3 Mice

Pretreatment of mice	/ heart		/ liver		/ lung		/ spleen		/ kidney		/ brain		Muscles of thighs		d l		c r	
	Days		*Days*		*Days*		*Days*		*Days*		*Days*		*Days*		*Days*		*Days*	
	7	14	7	14	7	14	7	14	7	14	7	14	7	14	7	14	7	14
Controls	⅓	0	71	1	369	1	8	1	⅓	0	0	0	0	0	3	2	8	⅓
Vaccinated	⅓	0	10	1	274	12	7	4	1	1	⅓	0	0	0	⅓	1	3	6
Infected 3 times	0	0	7	1	14	0	3	1	0	0	0	0	0	0	1	0	2	0

Larvae are obtained from the kidneys by *Baermann* technique—from the other organs by digestion in artificial gastric fluid

Key d. l = duodenum jejunum and ileum
c r = cecum colon and rectum

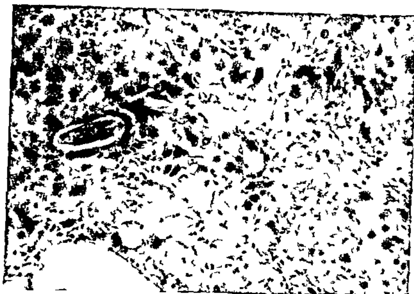


Fig 2

Liver from control mouse 7 days after challenge A larva not surrounded by cellular infiltration is seen to the left Partly necrotic parenchyma with haemorrhage to the right HE 160 ×

nature but in general lesions were found to be more numerous and damage more widespread in the controls

The first changes were seen on the 1st day after challenge and consisted of small scattered haemorrhages and small accumulations of lymphocytes Furthermore a few necrotic foci were found The cellular infiltration and the damage were steadily increasing and reached their maximum 5 to 6 days after challenge in vaccinated and re infected mice and 4 days after the test infection in the controls At the time of maximal reaction tortuous areas of necrotic parenchyma were frequently seen generally orientated from the portal tracts towards the central lobular veins but necrotic foci were also found throughout the parenchyma Accumulations of cells consisting predominantly of lymphocytes histiocytes heterophils and eosinophils were seen in relation to the portal tracts the central lobular veins and the necrotic tissue Sometimes the cells were infiltrating the necrotic areas but now and then necrotic tissue without any cellular reaction at all was seen

The lesions remained of constant severity for 4 to 5 days Then the cellular reaction would be associated intimately with the necrosis and the picture gave an impression of reparation Ten to 11 days after challenge the histological picture was almost normal

Larval remnants were observed 1 to 6 days after challenge in vaccinated mice 2 to 6 days after in re infected mice and 1 to 5 days after challenge in the controls It was remarkable in all 3 groups that only



Fig 3

Liver from resistant mouse 5 days after challenge Cross sections of larvae not surrounded by any cellular infiltration Necrotic tissue and haemorrhage in the middle HE 400 ×

few larvae were observed in association with cellular reactions In almost every case the larvae were found between apparently healthy hepatic cells or they were lying in a necrotic focus or surrounded by blood (Fig 2)

There was no tendency to greater anti larval activity in livers from resistant mice than in livers from non resistant mice (Fig 3) It was common to the 3 groups that the larvae found in the livers on the 4th day and onwards after challenge apparently were third stage larvae

Only very few cells stained positively with pyronin

Lung

Macroscopical Appearance From the 2nd or 3rd day after challenge an increasing number of pin point haemorrhages were seen on the surface of the lungs in vaccinated mice and controls True pneumonia was seen only in one mouse in the vaccinated group The lesions just described are quite the reverse of lung lesions in the re infected mice Here only very few haemorrhages were noticed but obvious inflammatory consolidation was seen in all specimens In 2 cases part of the tissue had turned into an abscess

Histology

Vaccinated mice and controls During the whole experimental period haemorrhages were seen in alveolar spaces and in septae but most

pronounced in the controls Until the 7th to 9th day after *challenge* the cellular reaction was minimal and limited to small concentrations of macrophages and lymphocytes in relation to bronchi and vessels For the *rest of the period of observation* extensive haemorrhages and cellular infiltrations were predominant The lesions became larger and tended to become confluent The cellular picture was similar in the 2 groups Lymphocytes histiocytes neutrophils eosinophils and some plasmacells accumulated in septae alveolar spaces *and particularly* around bronchi and vessels Until the 8th or 9th day after challenge only a very small number of pyronin stained cells was seen in these 2 groups From that time a moderate number was observed particularly in relation to vessels and branches of bronchi The lesions were obviously a combination of a moderate pneumonitis and a bronchopneumonia Desquamation of the epithelium of the alveolar sacs and *the bronchi was frequently seen*

Sometimes small infiltrations with granulocytes were observed below the intima of the blood vessels which were often surrounded by edema and dilated lymphatic vessels with some granulocytes

Larvae were found from the 5th to 11th day after challenge They were almost in every case lying without any association to the inflammatory reactions The maximum number of larvae was seen on the 7th day Sometimes the intestinal lumen of the larvae was packed with erythrocytes The size of larvae from vaccinated mice and from controls did apparently not differ

Re infected mice The histological lesions in all the lungs were of *almost similar nature from the day before challenge to the end of the* experimental period The inflammatory reaction was very severe and resulted in marked consolidation of the tissue (Fig 4) Haemorrhages were minimal The cellular reaction was extremely marked *in relation* to the bronchi and the blood vessels (Fig 5) but also septae and the alveolar spaces were heavily infiltrated with large numbers of lymphocytes histiocytes a few giantcells eosinophils neutrophils and with plasmacells and primitive blastlike cells The pyroninophilia differed markedly from those in the preceding groups Already from day zero there was a large number of positively stained cells among which there were many true plasmacells The cells were especially accumulating in enormous numbers around vessels and bronchi whereas the cells were poorly represented in the septae

There was a pronounced desquamation of the epithelium of the *alveolar sacs and the bronchi* and many macrophages contained phagocytized granulocytes The alveolar sacs sometimes contained a fibrin like substance and the lumen of the branches of bronchi was packed with immense numbers of *granulocytes but did not* contain pyronin stained cells The epithelium of the bronchi was hypertrophic and septae of connective tissue covered by epithelium seemed to protrude into the lumen The areas of normal tissue were *minimal* in the speci



Fig 4

Lung section from resistant mouse The tissue is heavily infiltrated with granulocytes lymphocytes and macrophages HE 160 ×

mens examined Granulocytes were seen in the wall of the vessels and below the intima

Larvae were not detected in any section

Spleen

Macroscopical appearance The spleens from all 3 groups varied very much in size but nothing seemed to indicate that enlarged spleens were more numerous in one group than in another

Histology There was no conspicuous difference between the spleens from the 3 groups as detected by examination of sections stained with HE AG and MP Follicles were always present and the red pulp was congested

DISCUSSION AND CONCLUSION

The course of migration in the controls—maximum number of larvae in the liver and lungs 2 and 7 days after challenge respectively—is largely in accordance with observations in mice by previous authors (*Sprent & Chen* 1949 *Sprent* 1952 *Bhowmick* 1964 *Sinha* 1967)

It appears from Fig 1 that the course of migration in vaccinated and re infected mice differs somewhat from that in the controls It is common to the 2 resistant groups that establishment of larvae in the liver is redused on the first day following challenge as compared to the controls and that only about half as many larvae as in the controls are getting to the liver at all



Fig 3

Lung section from resistant mouse Heavy cellular accumulation surrounding a small vessel A great number of the cells stained with pyronin HE 160 ×

This divergence in the early migration of larvae must be taken as an expression of an apparent host response in resistant mice against larvae induced by the immunization procedures preceding challenge The defence mechanism provoked in the host is obviously not only responsible for a mere retardation of larval penetration through the gut wall but also for a remarkable degree of larval destruction However the present study gives no information concerning the exact site of *action of the host response whether it acts in relation to hatching* of eggs penetration of intestinal mucosa or to the very passage through the gut wall

It is shown in Fig 1 that the total number of larvae in vaccinated animals was about half the number of larvae in the controls and that the larvae which once had penetrated the gut wall behaved like larvae in the controls This suggestion is confirmed by the histological examination which gives no evidence of an increased host reaction in the livers of vaccinated mice compared to the controls Thus the preceding results confirm the previous observations by the present author (*Bindseil* 1969) that some protection against a challenge infection is obtainable by re injection procedures *using adult Ascaris extract* Furthermore the results show that the effect seems to exert an action only in relation to the gut

An apparent delay of passage and a destruction of larvae in the intestine are also characteristic features of the early larval migration in re infected mice The minimal number of larvae recovered from the lungs of these mice might indicate a significant larval destruction in

the liver but the histological examination of the livers did not reveal any accelerated host tissue reaction Furthermore the livers did not even histologically reflect the 3 previous infections

Undoubtedly the reason why the damages in the livers of the controls are more pronounced than those in the immune groups is that much more larvae have migrated through the livers

In the search for reasons why the establishment of larvae in the lungs is reduced in re infected mice it is reasonable to turn the attention to the serious and widely spread pathological findings in the lungs from these animals The pattern of a possible host response against larvae at that site is however obscure The pathological findings are as manifest the day prior to challenge as at the end of the experimental period so it is obvious that the larvae from the challenge dose have not participated very much in the development of the changes but possibly they may have participated in their maintenance That the tissue reaction may all the same indicate a specific host response of a possible immunological nature can be motivated by the large numbers of pyronimophils found in the lungs suggesting some increased antibody activity One might also suggest that the mechanism is purely unspecific and say that the heavily inflammated tissue is a very unfavourable habitat for the larvae and that they would succumb due to physical chemical changes in the tissue thereby causing deterioration of the environment

The figures presented in Table 1 show convincingly that an establishment of larvae at other sites in vaccinated and re infected mice due to the fact that a deterioration of the normal habitat does not take place and thus does not account for the reduced number of larvae recovered from the normal sites of predilection

As mentioned most previous authors have claimed that protective immunity against *Ascaris suum* is predominantly working in the liver The present study does not confirm these statements but shows that the immune response (resistance against challenge) is primarily working in relation to the gut Thus the study confirms the findings by Wagner (1933) who did not ascribe any importance to the liver as the site of action Acquired immunity to *Ascaris suum* in guinea pigs is studied in detail by *Soulsby* (1961) who claims that the immunological host response in immune animals is not active until the first moulting process in the liver sets in about 5 days following challenge or in other words that the larvae must progress to a certain point of development before such antigens as function in resistance are liberated in the host

The different results obtained during the experiments on *Ascaris suum* force one to consider the possibility that the mechanism of resistance against a parasite may operate at different sites in different hosts

SUMMARY

The present study was carried out to investigate the fate of *Ascaris suum* larvae in resistant mice The mice were made resistant by re injections of extract from adult *Ascaris suum* and by re infections with infective *eggs of the parasite*

Immune mice and controls were sacrificed daily and larval counts and histological examinations were *carried out on liver and lung*

It was found that the defence mechanism in either groups of im mune mice was only operating in the intestine and no indication was found that the liver was participating in the defence mechanism as stressed by most previous authors

As regards the re infected mice the lungs were considered a pos sible second site of action of the host response and it was discussed whether the mechanism was *purely unspecific or it was based upon a* specific immunological response

REFERENCES

1 *Bhowmick D K* Beiträge zu dem Problem der Wanderwege der Askariden larven (*Ascaris Lumbricoides Linné* 1758 und *Toxocara canis Werner* 1782) im experimentellen und naturlichen Wirt Z Parasitkde 24 121–168 1964

2 *Bindseil E* Immunity to *Ascaris suum* 1 Immunity induced in mice by means of material from adult worms Acta path microbiol scand 77 218–222 1969

3 *Brambell M R* Effects of the age of infestation and of the immunity of the host on the distribution of *Nippostrongylus brasiliensis* in the small in testine of rats Parasitology 53 7 P 1963

4 *Casarosa L* Personal communication 1967

5 *Fallis A M* Resistance to *Ascaris lumbricoides* L infection as demonstrated experimentally in guinea pigs Can J publ Hlth 35 90 1944

6 *Fallis A M* *Ascaris lumbricoides* infection in guinea pigs with special refer ence to eosinophilia and resistance Can J Res 26 307–327 1948

7 *Kelley G W & Nayak D P* Acquired immunity to migrating larvae of *Ascaris suum* induced in pigs by repeated oral inoculation of infective eggs J Parasit 50 499 503 1964

8 *Kelley G W & Nayak D P* *Passive immunity to Ascaris suum transferred in* colostrum from sows to their offspring Am J vet Res 26 *948–950* 1965

9 *Kerr K B* The cellular response in acquired resistance in guinea pigs to an infection with pig *Ascaris* Am J Hyg 27 28–51 1938

10 *Matoff K & Tersijski A* Besteht eine Darmphase der erworbenen Immunität gegen *Ascaris suum*? Z Tropenmed Parasit *18* 343–353 1967

11 *Michel J F* Immunity to helminths associated with the tissues Sixth Symp Brit Soc Parasit 67–89 1968

12 *Sinha B N* The migratory behaviour of the larvae of *Ascaris lumbricoides* (*Linnaeus* 1758) in white mice Indian vet J 44 292–294 1967

13 *Soulsby E J L* Some aspects of the mechanism of immunity to helminths J Am vet med Ass *138* 355–362 1961

14 *Sprent J F A* On the migratory behavior of the larvae *of various Ascaris* species in white mice J infect Dis *90* 165–176 1952

15 *Sprent J F A & Chen H H* Immunological studies in mice infected with the larvae of *Ascaris lumbricoides* I Criteria of immunity and immunizing effect of isolated worm tissues J infect Dis *84* 111–124 *1949*

16 *Taffs L F* Immunological studies on *experimental infection of pigs* with *Ascaris suum Goeze 1782 VI The histopathology of the* liver and lun J Helminth 42 157–172 1968

17 *Wagner O* Immunisierungsversuche bei experimenteller *Askaris Infektion* der Maus Z ImmunForsch exp Ther *78* 372–382 *1933*

Acta path microbiol scand 77 235-246 1969

Laboratory of Oral Microbiology, Department of Microbiology The Gade Institute and Institute of Periodontology Schools of Medicine and Dentistry University of Bergen Norway

IMMUNOCHEMICAL STUDIES OF ORAL FUSOBACTERIA

1 *Major precipitinogens*

By

TORE KRISTOFFERSEN

Received 14 III 69

The identity of the genus *Fusobacterium* (23) has been established through a considerable body of research (3 5 6 7 12 13), although some confusion concerning its taxonomic status still remains Reports on the immunochemistry and antigenic structure of fusobacteria are scarce however

Weiss & Mercado (26) on the basis of precipitin tests with extracts from four different strains reported the presence of type specific proteins and group specific carbohydrates *de Araujo et al* (2) employing the technic of indirect hemagglutination with supernatants of heat killed bacteria as antigens found a broad cross reactivity among their strains of fusobacteria They found the antigen or antigens responsible for this cross reactivity to be of protein nature Purified lipopolysaccharides with endotoxic activity exhibited a high degree of serological specificity in hemagglutination inhibition tests and several serological types of fusobacteria could be distinguished (2) The antigen responsible for the broad cross reactivity was thought to form one of three precipitation bands observed with crude extract of strain ATCC 10 953 and antiserum to this strain When the antiserum was absorbed with purified lipopolysaccharide only this band was formed in agar double diffusion tests (2)

Investigations on the immunochemistry and antigenic structure of oral fusobacteria will be reported in a series of publications The present paper deals with major precipitinogens

MATERIALS AND METHODS

Isolation of oral fusobacteria Strains of fusobacteria were isolated from saliva or gingival scrapings from individuals exhibiting varying degrees of marginal periodontal disease Isolations were made from a modification of the medium of *Baird*

These investigations have been supported by a grant from *L Meltzers Høyskolefond*

Parker (4) containing 1 30 000 ethyl violet or from the medium proposed by *Omata & Disraely* (19) Plates were incubated at 37 C for 3 days in anaerobic jars with 7 per cent carbon dioxide in hydrogen After isolation the strains were grown in Brain Heart Infusion broth (Difco) and lyophilized or they were maintained in a fluid thioglycollate medium (Difco) Seventeen strains were isolated in this manner

The *Fusobacterium polymorphum* strains 1T3 and Fevl were obtained through the courtesy of Dr Stephan E Mergenhagen Bethesda Maryland U S A The type strain ATCC 10 953 (American Type Culture Collection) was also included in the study

Fermentation studies were carried out largely as described by *Omata & Braunberg* (20) All strains corresponded morphologically and biochemically to *F polymorphum* Knorr or *F nucleatum* Knorr as described by *Omata & Braunberg* (20)

Mass cultivation took place in Brain Heart Infusion broth (Difco) usually for 4 days After centrifugation the organisms were washed twice with sterile distilled water and stored as a paste at —25 C until use

Rabbit immune sera against 11 strains of fusobacteria were prepared by intravenous injection of formalin killed microorganisms according to the injection scheme described by *Oeding* (18) The suspensions contained approximately 2 mg (dry weight) of microorganisms per ml In some instances suspensions of live microorganisms were used Samples of sera were obtained before immunization and were controlled by agar double diffusion not to contain precipitating antibodies to extracts from fusobacteria Antisera were stored at —25 C

Agar double diffusion tests were performed in glass petri dishes 9 cm in diameter Ten ml of 1 0 per cent agar (Special Agar Double Difco) in saline were poured into each dish One central and six peripheral circular wells 4 5 mm in diameter and 5 mm apart were cut in the agar with a special punching device. The wells were filled to capacity with antigen or antiserum Diffusion and precipitation usually took place at room temperature and readings were made after one two and three days

Ring test precipitation was carried out and read as described by *Haukenes et al* (11)

Disruption of bacteria The X Press (8) (A/B BIOS Nacka Sweden) was used for crushing of washed bacteria After 5 passages no intact cells could be seen in Gram stained smears

Extraction procedures In preliminary studies the efficiency of different extraction procedures were studied by agar double diffusion tests Crushing of microorganisms prior to extraction gave better results than extraction from undisrupted cells A variety of buffers with different salt concentrations and with pH ranging from 5 2 to 8 6 were tried at different temperatures Since the efficiency of the extraction did not vary to any great extent with the different buffers employed 0 05 M phosphate buffer pH 7 4 was used Unless otherwise stated buffer extractions were performed with occasional stirring for 48 hours at 4 C employing 10 ml of buffer to one gram (wet weight) of crushed bacteria Supernatants were collected after centrifugation at 25 000 × *g* at 4 C (crude extract)

Crushed bacteria were also extracted by the phenol water procedure of *Westphal & Lüderitz* (27) as outlined by *de Araujo et al* (?) Extraction was carried out for 15 minutes at room temperature with constant stirring The mixture was centrifuged at 9 000 × *g* for 30 minutes After the aqueous phase had been pipetted off an equal amount of distilled water was added and the procedure was repeated The combined aqueous phases were dialyzed exhaustively against distilled water The slightly opalescent extract could be stored at 4 C for several weeks without loss of serological activities (phenol water extract) The precipitate obtained when 3 volumes of ethanol were added to the phenol phase was dialyzed exhaustively and lyophilized The material was nearly insoluble in water and showed no precipitating ability nor was it active in absorption experiments

Enzymatic digestion of extracts Digestion with trypsin (crystalline Trypure Novo) α chymotrypsin (ex bovine pancreas crystallized Sigma) and pronase (B Grade Calbiochem) was performed in 0 05 M phosphate buffer pH 7 3 or in 0 0? M Tris HCl buffer pH 7 6 Papain digestion (2 × crystallized Sigma) was carried out in 0 1 M phosphate buffer pH 7 4 in the presence of 0 01 M cysteine and 0 002 M ethylenediaminetetraacetate Digestion with pepsin took place in 0 05 M acetate

buffer pH 4 1 After digestion with pepsin the material was neutralized by brief dialysis against buffered saline

For pronase the protein enzyme ratio (w w) was 20 1 or 10 1 For the remaining proteolytic enzymes different protein enzyme ratios were used varying from 50 1 to 100 1

As a control of activity of the different proteolytic enzymes their effect on coagulated egg white was observed visually In order to test for the possible occurrence of enzyme inhibitors in the extracts digestion of pieces of coagulated egg white was allowed to take place in the presence of the test substrate in appropriate concentrations No inhibitory effect was noted

Treatment with lysozyme took place in 0 05 M phosphate buffer pH 6 2 containing 0 1 M sodium chloride Fifty μg of crystalline egg white lysozyme (Koch Light) was added to a test tube containing 1 mg (dry weight) of extract in 1 ml The activity of the enzyme was tested by observing its lytic effect on cells of *Micrococcus lysodeikticus* (NCTC 2665)

All enzymatic digestions took place at 37 C for 18 hours Controls without enzyme were always included

For *periodate oxidations* of crude extracts a final concentration of 0 05 M Na *meta* periodate in 0 05 M phosphate buffer pH 7 2 was used The reaction was allowed to proceed for 18 hours at room temperature in the dark Test solutions and appropriate controls were then dialyzed against phosphate buffered saline pH 7 2 for 4 hours

Electrophoresis Equipment type LKB 3276 B (LKB Produkter AB Stockholm Sweden) was used for paper electrophoresis with a conversion set 6800 A 2 for immunoelectrophoresis For paper electrophoresis a veronal buffer pH 8 6 ionic strength 0 1 and an acetate buffer pH 5 6 ionic strength 0 1 were used applying a current of 200 V for 16 hours Paper strips were stained with amido black or by the periodic acid Schiff (P A S) method as described in (24) Some strips were dried at room temperature and cut into 1 cm wide segments at a right angle to the direction of the electrophoretic run The segments were eluted with buffered saline and the eluates examined by agar double diffusion or ring test precipitation For immunoelectrophoresis a veronal buffer pH 8 6 ionic strength 0 05 and a 1 per cent agar were employed. Electrophoresis was run for 60 or 75 minutes at 250 V After application of antisera the plates were incubated at room temperature for at least 24 hours

Epinephrine skin tests were carried out as described by *de Araujo et al* (2) The experimental animals received a control intradermal injection of 0 1 ml of saline and control animals were given saline as a preparatory injection The rabbits used weighed 3 2 to 3 4 kg

Preparative ultracentrifugation was performed in a Beckman Spinco Model L ultracentrifuge in 10 ml tubes

For *gel filtration experiments* with Sephadex G 25 G 50 G 75 and G 200 (AB Pharmacia Uppsala Sweden) a 0 05 M phosphate buffer pH 7 4 was used Columns 2 5–3 0 cm in diameter and 30–40 cm high were packed largely as described by *Flodin* (9) and run at room temperature The rate of flow was approximately 20 ml per hour The void volume (V_0) was determined by passage of india ink through the columns

Protein was determined by the method of *Lowry et al* (15)

EXPERIMENTS AND RESULTS

Major Precipitation Lines

Buffer extracts of all strains gave positive ring tests with all antisera indicating a high degree of cross reactivity between the strains Cross reactions were also observed in agar double diffusion tests i e "crude extracts from one organism gave lines of precipitation with heterologous antisera and several strains or their extracts gave precipitation lines showing "reactions of identity or "reactions of partial identity" (21) with the same antiserum

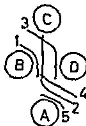


Fig 1

Schematic drawing of agar precipitation patterns showing principal lines formed with extracts from *Fusobacterium* strain F1 Antiserum strain F1 in well B Antiserum strain Fev1 in well A Wells C and D contained phenol water extract and buffer extract from strain F1 respectively Figures indicate principal lines as described in the text.

In order to simplify further experimental work one strain F1 was selected for closer examination Strain F1 was chosen because it is a typical *Fusobacterium* by the criteria used in this study It could easily be mass cultivated and antisera with high precipitation titres were obtained when this strain was used for immunization In addition it showed a strong cross reactivity in precipitin reactions with most of the other strains

Three major precipitation lines could be distinguished when crude extract of strain F1 was used as antigen in agar double diffusion with the corresponding antiserum (Fig 1) No lines were formed when concentrates of the media employed for cultivation of the organisms were used as antigens nor were the precipitins responsible for the lines absorbed from the sera by such concentrates The lines closest to the well containing antiserum developed rapidly and was sharp and well defined (line 1 Fig 1) The line corresponding to the most slowly diffusing precipitinogen (line 3 Fig 1) and the center line (line 2 Fig 1) developed more slowly Phenol water extract from crushed F1 microorganisms formed two lines with homologous antiserum These two lines fused with line 1 and line 3 respectively When antiserum to strain F1 was absorbed with an excess amount of "phenol water extract only the center line (line 2 Fig 1) was formed in agar with crude extracts

In addition to the three major lines seen with antiserum to formalin killed F1 bacteria strain F1 antisera to live F1 and several other strains of fusobacteria gave another line with crude extract from strain F1 (line 4 Fig 1)

With antisera to two of the strains the presence in extracts from strain F1 of a rapidly diffusing precipitinogen different from that which gave rise to line 1 could be demonstrated (line 5 Fig 1) With antiserum to strain F1 this line was usually masked out by the heavier line 1 It could however be demonstrated by immunoelectrophoresis (Fig 3 A)

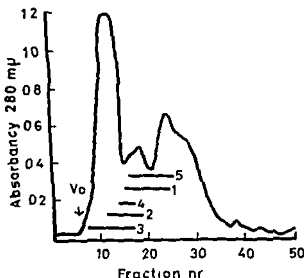


Fig 2

Gel filtration of 50 mg lyophilized buffer extract strain F1 Column 50 × 2 5 cm packed with Sephadex G 200 0 05 M phosphate buffer pH 7 4 fractions 10 ml Horizontal lines 1 to 5 lines on agar double diffusion V_0 = void volume

The distribution among strains of fusobacteria of the precipitinogens responsible for the five precipitation lines described has not been extensively studied However extracts from all of 20 strains investigated so far when tested against antiserum to strain F1 formed a line giving a reaction of identity with line 2 Inversely this line could be demonstrated with all of our 11 antisera

A number of experiments have been carried out in order to further characterize the precipitinogens corresponding to the precipitation lines that have been described For the sake of brevity the substances giving rise to lines 1 2 3 4 and 5 will henceforth be termed Precipitinogens 1 2 3 4 and 5 respectively

Preparative Ultracentrifugation and Tests for Endotoxic Activity

The dialyzed aqueous phase from phenol water extractions was ultracentrifuged for 1 hour at 100 000 × *g* The supernatant formed only line 1 with the homologous antiserum The precipitate was washed twice in distilled water with ultracentrifugation at 100 000 × *g* each time after which it was suspended in distilled water and lyophilized The product gave a slightly opaque suspension in aqueous media at neutral pH Only line 3 was formed in agar with homologous antiserum when a suspension containing 1 mg/ml of the lyophilized precipitate was used as antigen A faint line corresponding to line 1 was visible with concentrations higher than 10 mg/ml In epinephrine

skin tests 50 µg of this material gave a positive reaction in both rabbits tested Controls were negative

Gel Filtration

All of the five precipitinogens appeared in a small elution volume in gel filtration experiments when 10 ml of unconcentrated crude extract was applied to a column 10 cm high and with an internal diameter of 2 5 cm packed with Sephadex G 25 or Sephadex G 50 The precipitinogens giving rise to lines 1 and 5 were slightly retained on a similar column packed with Sephadex G 100

Results obtained in a typical experiment with a column 2 5 × 50 cm packed with Sephadex G 200 are illustrated in Fig 2 The sample applied to the column was 50 mg of dialyzed and lyophilized crude extract Reducing the size of the sample did not appreciably improve the separation When serial dilutions of the fractions were used in agar double diffusion tests a partial separation of all of the precipitinogens could be demonstrated The effluent volumes of the highest concentrations of the five precipitinogens as determined by agar precipitation titres were approximately

Precipitinogen	1	$3\,4 \times V_0$
"	2	$2\,0 \times V_0$
	3	$1\,2 \times V_0$
	4	$2\,5 \times V_0$
	5	$4\,0 \times V_0$

Electrophoresis

The identity of the precipitation bands on immunoelectrophoresis could be established through the use of absorbed antisera and by *immunoelectrophoretic* analysis of fractions obtained by gel filtration on Sephadex G 200

Immunoelectrophoresis of crude extract at pH 8 6 gave a good separation of the Precipitinogens 2 4 and 5 from each other and from Precipitinogens 1 and 3 (Fig 3 A) The band corresponding to line 3 was usually very faint in immunoelectrophoresis of buffer extracts

When the phenol water extract was subjected to immunoelectrophoresis (Fig 3 B) the two bands corresponding to lines 1 and 3 showed that the corresponding precipitinogens had moved towards the anode under the experimental conditions

The band *corresponding* to line 3 showed a tendency to split *into* two curves which fused completely This tendency was more *clearly* evident when the material responsible for this band had *been concentrated* by ultracentrifugation *prior to immunoelectrophoresis* (Fig 3 B and C)

No separation of Precipitinogens 1 and 3 was achieved on immunoelectrophoresis nor could they be separated by paper *electrophoresis*

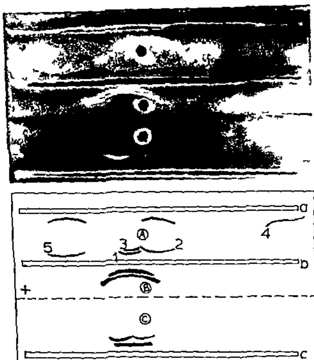


Fig 3

Immunoelectrophoretic patterns with extracts of fusobacteria strain F1
Buffer extract
Aqueous phase from phenol water extractions 3 mg per ml
Precipitate from ultracentrifugation of aqueous phase from phenol water extract 20 mg per ml
oughs b and c contained antiserum strain F1 Trough a contained antiserum strain Fev1 Code for line see Fig 1

pH 8 6 or pH 5 6 of phenol water extract Both precipitinogens orresponded to a wide and strongly P A.S positive band moving to ards the anode with both electrophoresis buffers A small amount f material staining with amido black remained at or near the line f application

The concentrations of the five precipitinogens in the crude extract were too low for these substances to be conclusively localized in paper trips after electrophoresis

Effects of Various Treatments on the Precipitinogens

Crude extract which had been heated at 100 C for 1 hour at pH 7 4 precipitated only lines 1 and 3 in agar Lines 2 4 and 5 could not be demonstrated

The supernatant obtained after heating 1 g of whole cells strain ATCC 10 953 in 5 ml of 0 05 M phosphate buffer pH 7 4 at 100 C for

skin tests 50 µg of this material gave a positive reaction in both rabbits tested Controls were negative

Gel Filtration

All of the five precipitinogens appeared in a small elution volume in gel filtration experiments when 10 ml of unconcentrated crude extract was applied to a column 40 cm high and with an internal diameter of 2 5 cm packed with Sephadex G 25 or Sephadex G 50 The precipitinogens giving rise to lines 1 and 5 were slightly retained on a similar column packed with Sephadex G 100

Results obtained in a typical experiment with a column 2 5 × 50 cm packed with Sephadex G 200 are illustrated in Fig 2 The sample applied to the column was 50 mg of dialyzed and lyophilized crude extract Reducing the size of the sample did not appreciably improve the separation When serial dilutions of the fractions were used in agar double diffusion tests a partial separation of all of the precipitinogens could be demonstrated The effluent volumes of the highest concentrations of the five precipitinogens as determined by agar precipitation titres were approximately

Precipitinogen 1	3 4 × V_o
2	?0 × V_o
3	1? × V_o
4	2 5 × V_o
5	4 0 × V_o

Electrophoresis

The identity of the precipitation bands on immunoelec could be established through the use of absorbed antisei immunoelectrophoretic analysis of fractions obtained by ge on Sephadex G 200

Immunoelectrophoresis of crude extract at pH 8 6 ga separation of the Precipitinogens 2 4 and 5 from each from Precipitinogens 1 and 3 (Fig 3 A) The band corr to line 3 was usually very faint in immunoelectrophoresis extracts

When the phenol water extract was subjected to immu phoresis (Fig 3 B) the two bands corresponding to lines showed that the corresponding precipitinogens had movec the anode under the experimental conditions

The band corresponding to line 3 showed a tendency to split into two curves which fused completely This tendency was more clearly evident when the material responsible for this band had been concentrated by ultracentrifugation prior to immunoelectrophoresis (Fig 3 B and C)

No separation of Precipitinogens 1 and 3 was achieved on immuno electrophoresis nor could they be separated by paper electrophoresis

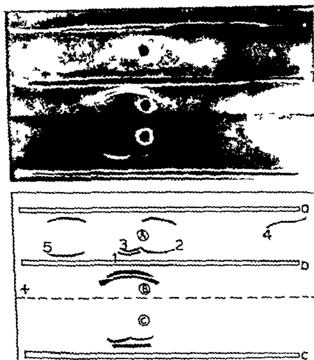


Fig 3

Immunoelectrophoretic patterns with extracts of fusobacteria strain F1
A Buffer extract.
B Aqueous phase from phenol water extractions 3 mg per ml
C. Precipitate from ultracentrifugation of aqueous phase from phenol water extract 20 mg per ml
Troughs b and c contained antiserum strain F1 Trough a contained antiserum strain Fev1 Code for line see Fig 1

at pH 8 6 or pH 5 6 of phenol water extract Both precipitinogens corresponded to a wide and strongly P A S positive band moving towards the anode with both electrophoresis buffers A small amount of material staining with amido black remained at or near the line of application

The concentrations of the five precipitinogens in the crude extract were too low for these substances to be conclusively localized in paper strips after electrophoresis

Effects of Various Treatments on the Precipitinogens

Crude extract which had been heated at 100 C for 1 hour at pH 7 4 precipitated only lines 1 and 3 in agar Lines 2 4 and 5 could not be demonstrated

The supernatant obtained after heating 1 g of whole cells strain ATCC 10 953 in 5 ml of 0 05 M phosphate buffer pH 7 4 at 100 C for

one hour gave no precipitation in agar or ring test with antiserum to strain F1

Equal volumes of crude extract and 90 per cent phenol were mixed for 15 minutes at room temperature. The dialyzed aqueous phase obtained after centrifugation formed only lines 1 and 3 in agar. The phenol phase was precipitated with 3 volumes of ethanol. The precipitate was suspended in distilled water, dialyzed overnight and dried in a desiccator. A suspension containing 10 mg/ml gave no precipitation lines in agar.

Periodate oxidation destroyed the precipitating ability of Precipitinogens 1, 2 and 3. Precipitinogens 4 and 5 remained seemingly unaffected by this treatment.

None of the precipitinogens were affected by digestion with lysozyme, trypsin, α-chymotrypsin or papain for 18 hours. Digestion with pronase and pepsin destroyed Precipitinogens 2, 4 and 5 while the agar precipitation titres with respect to Precipitinogens 1 and 3 remained unchanged. The effects on the precipitating ability in agar of various treatments of buffer extract have been compiled in Table 1.

TABLE 1
Effect on the Precipitating Ability in Agar of Various Treatments of Crude Extracts from Crushed Fusobacteria Strain F1

Precipitinogen	Pepsin	Pronase	Periodate	Heat	Phenol
1	—	—	+	—	—
2	+	+	+	+	+
3	—	—	+	—	—
4	+	+	—	+	+
5	+	+	—	+	+

\+ Precipitating ability in agar destroyed by treatment
— Precipitating ability in agar seemingly unaffected by treatment
Trypsin, papain, α-chymotrypsin and lysozyme did not affect the precipitating ability of the precipitinogens

Mild Acid Hydrolysis of Phenol Water Extracts

Aliquots of the dialyzed aqueous phase after phenol water extraction containing 3 mg of dry material per ml were subjected to hydrolysis at 100 C for 2 and for 7 minutes in 0.1 N acetic acid. After hydrolysis the suspensions were neutralized and the volume was adjusted to 3 ml with a neutral buffer. Serial dilutions of the hydrolyzed samples and of an unhydrolyzed sample containing 1 mg/ml of dry material were used as antigens in agar double diffusion tests against an antiserum to strain F1. Results are recorded in Table 2 as the reciprocal of the highest dilutions of hydrolyzed and unhydrolyzed samples precipitating the respective lines in agar with homologous antiserum

TABLE 2

Effect of Brief Acid Hydrolysis of Dialyzed Aqueous Phase from Phenol Water Extractions

Precipitation line	Highest dilution of antigen giving precipitation with homologous antiserum		
	Unhydrolyzed	Hydrolyzed 2 min	Hydrolyzed 7 min
3	4096	256	1
1	8	256	1024

Hydrolysis gave a marked reduction in the amount of material precipitating line 3. After hydrolysis for 7 minutes this line was barely visible with undiluted test sample. At the same time the concentration of material precipitating line 1 was increased approximately 128 times.

DISCUSSION

The cross reactivity on ring test and agar precipitation of all strains of fusobacteria included in this study is in accordance with the findings of *Boe* (7) and parallels the findings of *de Araujo et al.* (2).

Precipitinogens 2, 4 and 5 could be separated by electrophoresis or gel filtration or both. The precipitation lines in agar double diffusion and immunoelectrophoresis gave no indication of a serological relationship between the 3 precipitinogens. The conclusion therefore seems justified that precipitation lines 2, 4 and 5 are caused by different substances.

The relationship between Precipitinogens 1 and 3 is less obvious. Although no chemical data are presented, it can be concluded from other findings that the material giving rise to line 3 corresponds to the endotoxic lipopolysaccharide antigen. The presence of such materials in fusobacteria was suggested by *Boe* (7) and confirmed by *Hergenhagen et al.* (16) and by *de Araujo et al.* (2). The endotoxin preparations of *de Araujo et al.* (2) formed two lines in agar. Similar observations have been reported by *Ribi et al.* (22) with endotoxins prepared by different methods from various *Enterobacteriaceae*. The component moving most rapidly through the gel was found by *Ribi et al.* (22) to be formed as a degradation product by mild acid hydrolysis of endotoxin from *Salmonella enteritidis* and to be serologically indistinguishable from the "acid hapten" extractable by a modified Freeman (10) procedure. The results of mild acid hydrolysis of our phenol water extract parallel the findings of *Ribi et al.* (22). This observation provides strong evidence that the material precipitating line 1 is analogous to the polysaccharide haptens found in *Enterobacteriaceae*. By absorption experiments *Ribi et al.* (22) demonstrated that endotoxin and hapten had serological specificities in

common These findings were in agreement with those of *Staub* (25) and of *Landy* (14) Preliminary absorption experiments with extracts from *Fusobacterium* strain F1 have indicated that there is a similar serological relationship between Precipitinogens 1 and 3

Heterogeneity in preparations of endotoxic lipopolysaccharides has been demonstrated by several methods such as ultracentrifugal analysis free boundary electrophoresis and more recently (17) by ion exchange chromatography The heterogeneity of Precipitinogen 3 demonstrated by immunoelectrophoresis of our preparation therefore was not surprising

In spite of the failure of some proteolytic enzymes to destroy the precipitating ability of Precipitinogens 2 4 and 5 a number of observations including their lability to heat and their destruction by phenol and by digestion with pepsin and pronase suggest that protein constituents are essential for the precipitating abilities of these substances

The precipitating ability of Precipitinogen 2 was destroyed by periodate as well as by pepsin and pronase This may suggest the presence of a carbohydrate component in addition to protein

Gel filtration may become useful in purification experiments Under certain circumstances and particularly with carbohydrate free globular proteins gel filtration may give valuable information about molecular weights (1) From the present experiments no conclusion can be drawn as to the molecular weights of the 5 precipitinogens

Our experiments so far have indicated that Precipitinogen 2 is quite widely distributed among oral fusobacteria The precipitinogen found in an extract of heat killed bacteria strain ATCC 10 953 by *de Araujo et al* (2) and thought to correspond to the antigen or antigens responsible for group reactivity in indirect hemagglutination experiments was reported to be destroyed by phenol The cross reactivity could not be demonstrated when the extract used for sensitizing sheep red blood cells had been subjected to tryptic digestion The effect of trypsin or other proteolytic enzymes on the precipitating ability of the extract was not reported Precipitinogen 2 described in the present study was heat labile and was not destroyed by tryptic digestion It therefore probably does not correspond to the antigen described by *de Araujo et al* (2)

SUMMARY

Major precipitinogens of oral fusobacteria were studied by agar double diffusion immunoelectrophoresis and ring test precipitation techniques

With extracts from one typical strain of fusobacteria 5 major precipitation lines were formed in agar Several observations indicated that two of these lines correspond to the endotoxic lipopolysaccharide and the so called acid hapten respectively

The precipitating ability of the antigens responsible for the remaining three lines were found to depend upon protein constituents One of them appeared to contain a carbohydrate component as well Although the distribution of the precipitinogens has not been extensively studied this latter precipitinogen appears to be very common among oral fusobacteria

REFERENCES

1 *Andrews P* The gel filtration behaviour of proteins related to their molecular weights over a wide range Biochem J *96* 595–606 1965

2 *Araujo W C de Varah E & Mergenhagen S F* Immunochemical analysis of human oral strains of *Fusobacterium* and *Leptotrichia* J Bact *86* 837–844 1963

3 *Araujo W C de* The specific status of *Fusiformis fusiformis* (Prévot) An Microbiol *11* 99–104 1963

4 *Baird Parker A C* Isolation of *Leptotrichia buccalis* and *Fusobacterium* species from oral material Nature (Lond) *180* 1056 1957

5 *Baird Parker A C* The classification of fusobacteria from the human mouth J gen Microbiol *2?* 458–469 1960

6 *B rger U* Untersuchungen an Fusobakterien I Mitteilung Systematik Zuchtung und Morphologie Zbl Bakt I Abt Orig *166* 484–497 1956

7 *Boe J* Fusobacterium Studies on its bacteriology serology and pathogenicity Skr norske Vidensl Akad I Mat nat Kl No 9 1941 p 191

8 *Edebo L* A new press for the disruption of microorganisms and other cells J bioch microbiol Technol Engng 2 453–470 1960

9 *Flodin P* Dextran gels and their applications in gel filtration Pharmacia Uppsala 196? Pp 42–48

10 *Freeman F G* The preparation and properties of a specific polysaccharide from Bact typhosum Ty Biochem J *36* 340 355 194?

11 *Haukenes G Losnegard N & Oeding P* Extraction and fractionation of antigenic material from *Staphylococcus aureus* Acta path microbiol scand *53* 84–93 1961

1? *Hine M K & Berry G P* Morphological and cultural studies of the Genus *Fusiformis* J Ba t *34* 517–534 1937

13 *Lahelle O* Necrobacterium A study of its ba teriolo y erology and pathogenicity and its relation to *Fusobacterium* Acta path microbiol cand Suppl 67 1947 Pp 361

14 *Landy M Johnson A G Webster M E & Sagin J F* Studies on the O antigen of *Salmonella typhosa* II Immunological properties of the purified antigen J Immunol *74* 466–478 195?

15 *Lowry O H Rosebrough N J Farr A L & Randall R J* Protein measurement with the Folin phenol reagent J biol Chem *193* 265–275 1951

16 *Mergenhagen S E Hampp E G & Scherp H W* Preparation and biological properties of endotoxins from oral bacteria J Infect Dis *108* 304–310 1961

17 *Nowotny A* Heterogeneity of endotoxic bacterial lipopolysaccharides revealed by ion exchange column chromatography Nature (Lond) *?10* 278–280 1966

18 *Oeding P* Agglutinability of pyogenic staphylococci at various conditions Acta path microbiol scand *41* 310–324 1957

19 *Omata R R & Disraely M N* A selective medium for oral fusobacteria J Bact. *7?* 677–680 1956

?0 *Omata R R & Braunberg R C* Oral fusobacteria J Bact *80* 737–740 1960

21 *Ouchterlony O* Diffusion in gel methods for immunological analysis Progress in Allergy *6* 30–154 (Karger Basel New York) 196?

?? *Ribi E Haskins W T Milner K C Anacker R L Ritter D B Goode G Trapani R J & Landy M* Physicochemical changes in endotoxin associated with loss of biological potency J Bact *84* 803–814 1962

23 *Rosebury T* Microorganisms indigenous to man McGraw Hill Book Co Inc New York 1962 Pp 144–185
24 *Smith I* Chromatographic and electrophoretic techniques Vol II Zone Electrophoresis Heinemann Medical Books Ltd and Interscience Publishers Inc London and New York, 1960 Pp 16–18
25 *Staub A M* Role des anticorps antipolyosidiques dans l agglutination des bacilles typhiques Ann Inst Pasteur 86 618–635 1954
26 *Weiss C & Mercado D G* Demonstration of type specific proteins in extracts of fusobacteria J exp Med 67 49–59 1938
27 *Westphal O* & *Luderit O* Chemische Erforschung von Lipopolysacchariden gramnegativer Bakterien Angew Chem 66 407–417 1954

Acta path microbiol scand 77 247-257 1969

Laboratory of Oral Microbiology Department of Microbiology The Gade Institute and Institute of Periodontology Schools of Medicine and Dentistry University of Bergen Norway

IMMUNOCHEMICAL STUDIES OF ORAL FUSOBACTERIA

2 *Some Properties of Undigested Cell Wall Preparations*

By

TORE KRISTOFFERSEN

Received 15 iii 69

Very little information about the composition and serological properties of cell walls of fusobacteria is available *Baird Parker* (5) reported some observations on the chemical composition of cell walls from six strains prepared by the method of *Cummins & Harris* (10) This procedure involves rather drastic treatment of the cells including digestion with proteolytic enzymes

In a preceding paper the five major precipitinogens found in one strain of *Fusobacterium* were described (27) Two of the precipitinogens were found to correspond to the endotoxic lipopolysaccharide and acid hapten respectively The remaining three precipitinogens were heat labile and were destroyed by 45 per cent phenol and by proteolytic enzymes One of them was found to be present in all of 20 strains of fusobacteria examined

The purpose of the present study was to investigate the composition of undigested cell walls from fusobacteria and the possible presence of the precipitinogens described (27) in such preparations

MATERIALS AND METHODS

Strains

The isolation and characterization of oral fusobacteria have been described (27) Three strains isolated in our laboratory *viz* strains F1 F2 and F4 were included in the present study In addition the type strain ATCC 10 953 (American Type Culture Collection) and one strain Fev1 kindly provided by Dr S E Mergenhagen Bethesda, Md were used

---

The author wishes to express his thanks to Dr K A *Selvig* Dental research laboratories University of Bergen School of Dentistry for carrying out the electron microscopic examinations

The quantitative *amino acid* analysis was performed by Mr *J Boe* Department of clinical biochemistry University of Bergen School of Medicine His help and advice are gratefully acknowledged

The investigations have been supported by grants from *L Meltzers Høyskolefond* and from the *Norwegian Research Council for Science and the Humanities*

Preparation of Cell Walls

Microorganisms were grown in Brain Heart Infusion broth (Difco) for 3–4 days harvested by centrifugation washed in saline and kept frozen until used

Cell walls were prepared essentially as recommended by *Yoshida et al* (50) The bacteria were crushed by five passages through the "X Press (17) A Servall Omni Mixer was used at low speed for 5 minutes to obtain even suspensions of crushed bacteria or cell wall fractions during preparation On the basis of pilot experiments for each preparation conditions for density gradient centrifugation were varied from 500 to 900 × *g* for one hour The cell wall fractions were washed thoroughly in distilled water and finally lyophilized

All preparations were examined for the presence of nucleic acid components by various chemical methods to be described presently In addition the cell wall preparation from strain F1 was studied by electron microscopy A suspension of cell walls was allowed to dry on grids previously covered with carbon substrates and shadowed with palladium at an angle of approximately 20 or they were stained with uranium acetate

Paper and Thin Layer Chromatography

Samples were hydrolyzed in glass stoppered tubes in

1 0 1 N HCl for 2 hours at 100 C
2 3 N HCl for 3 hours at 100 C
3 6 N HCl for 18 hours at 105 C or
4 1 N H_2SO_4 for 16 hours at 100 C

Acid was removed from the HCl hydrolysates by evaporation over NaOH pellets and the samples were taken up in measured amounts of distilled water The H_2SO_4 hydrolysates were neutralized and prepared for paper chromatography with special reference to heptoses as described by *Kauffmann et al* (26)

Circular paper chromatography was carried out as described by *Grov & Alsaker* (20) using Whatman no 1 paper For some chromatographic studies thin layer chromatography utilizing the Eastman Chromagram set with Eastman cellulose sheets 6065 was employed

The solvents used were

A	*Iso* propanol 2 N HCl (65 35)	(28)
B	BuOH HAc H O (4 1 1)	(36)
C	EtAc Py H O (12 5 4)	(47)
D	BuOH Py H O (6 4 3)	(24)
E	PhOH H O (4 1)	(9)
F	BuOH EtOH H O NH_3 d 0 88 (40 10 49 1 organic phase)	(19)

Solvent A was used for purines or pyrimidines solvents B and E for amino acids and amino sugars solvents C and D for sugars and amino sugars and solvent F for sugar alcohols

Chromatograms were stained with

1 Aniline hydrogen phthalate for aldoses (3a)
2 The alkaline silver nitrate reagent for reducing sugars (44)
3 The Elson Morgan reagents as recommended by *Smith* (41) for amino sugars
4 Na periodate benzidine for sugar alcohols (8)
5 Ninhydrin 0 4 per cent in acetone containing 2 per cent acetic acid for amino acids and amino sugars (31)
6 Isatine for proline and hydroxyproline (1)

Quantitative Analyses and Colorimetric Tests

Nitrogen was determined by the micro Kjeldahl technique according to the method described in (25) Samples were digested for 8 hours

Determination of *total phosphorus* was performed according to *Fiske & Subbarow* (18) with some of the modifications of *Youngburg & Youngburg* (51)

Neutral sugars were measured by the *Winzler* orcinol method (48) The compo

sition of the standards used was selected on the basis of results from chromatographic analyse Thus glucose was used as standard with cell walls from strains F1 F2 F4 and Fevl and glucose galactose (2 1) with cell walls from strain ATCC 10 953

Methylpentose was estimated by the cysteine sulphuric acid method as described by *Dische* (15) with rhamnose as standard The cysteine sulphuric acid reaction was also used as a test for *heptoses* (13)

Sialic acids were sought in samples hydrolyzed at 80 C for one hour in 0 1 N H_2SO_4 by the method of *Warren* (45) The modification of the direct Ehrlich method described by *Barry et al* (6) was also used In both tests N acetyl neuraminic acid was used as standard

The presence of *2 keto 3 deoxy sugar acids* was investigated by the method of *Weissbach & Hurwit* (46) in samples hydrolyzed in 0 02 N H_2SO_4 for 20 minutes at 100 C (34) *2 Deoxypentoses* were sought by the diphenylamine reaction (14)

Estimation of hexosamines wa performed by the *Rondle & Morgan method* (38) with glucosamine as standard

Fatty acid esters were estimated as tripalmitin by the method of *Snyder & Stephens* (42)

Quantitative analysis of *amino acids* was performed on a Beckman Spinco model 120 B Amino Acid Analyzer according to the method of *Moore et al* (32) and *Spackman et al* (43) The sample to be analyzed was hydrolyzed for 20 hours in 6 N HCl at 105 C and prepared for analysis as recommended in (16)

Other methods

The methods for production of *antisera* and *agar* and *ring test precipitation* were the same as described in (27) Buffer extracts" phenol water extracts and endotoxic lipopolysaccharide were al o produced as described (27)

EXPERIMENTS AND RESULTS

A *Appearance and Purity of Cell Wall Preparations*

The disintegrated bacteria had a marked tendency to clump during cell wall preparation Once a fairly homogeneous suspension could be obtained however a rather distinct layer of cell walls was formed in the sucrose gradient

The lyophilized preparations varied in colour from white to light grey Repeated washings in water or saline did not suffice to remove the greyish colour

The electron micrographs demonstrated that the disruption of the cells had been almost complete Only a few cells had retained a recognizable shape Mostly the walls appeared as thin irregular flakes The preparation examined also contained scattered electron dense round or irregular granules

Samples 0 3-0 4 mg of all preparations were hydrolyzed in 0 1N HCl and subjected to chromatography in solvent system A No spots related to nucleic acids were detected by examination of the dried chromatograms under a UV lamp One μg of adenine or guanine gave brightly flourescent spots No ribose was detected when 3N HCl hydrolysates were subjected to chromatography in solvent systems C or D Samples up to 0 7 mg were used The diphenylamine reaction gave a faint green colour with 1 3–1 6 mg amounts of the different preparations No absorption maximum at 595 mμ was observed

B Chemical Analyses

One cell wall preparation from each of the five strains was used for chemical analyses

On paper chromatographs glucosamine and small amounts of muramic acid were demonstrated in 3N HCl hydrolysates with the Elson Morgan reagents Glucosamine was also detected with the ninhydrin reagent in samples hydrolyzed in 6N HCl

Hydrolysates of cell walls from strains F1 F2 F4 and Fev1 gave strong spots corresponding to glucose Little or no galactose was detected With cell walls from strain ATCC 10 953 the spot corresponding to galactose was strong and much smaller amounts of glucose appeared to be present Rhamnose was present in all preparations and trace amounts of xylose were also found With location reagent 4 small amounts of glycerol were demonstrated

Samples hydrolyzed in 1N H_2SO_4 for 16 hours and subjected to circular paper chromatography in solvent system B gave a weak spot with location reagent 1 which on the grounds of the characteristic colour (22) and the Rf value (11) was tentatively ascribed to an aldoheptose The presence of heptose in our cell wall preparations was confirmed by the demonstration of a small but distinct absorption maximum at 510 mμ in the cysteine sulphuric acid reaction A sample 2 0 mg of endotoxic lipopolysaccharide from strain F1 gave a strong positive reaction The identity of the heptose has not been established It appeared from the chromatograms that the same and only one heptose was present in the five preparations

TABLE 1

Results of Quantitative Chemical Analyses of Cell Wall Preparations from Different Strains of Fusobacteria Per Cent of Dry Weight without Correction for Water Uptake during Hydrolysis Mean of 2–6 Determinations

Cell walls from strain	N per cent	P per cent	Neutral sugar per cent	Hexosamine per cent	Methyl pentose per cent	Fatty acid ester per cent
F1	10 24	0 53	5 6	9 2	1 2	11 [illegible]
F2	10 53	0 74	5 5	10 7	1 1	8 6
F4	10 97	0 48	5 3	9 8	0 9	10 [illegible]
Fev1	10 03	0 48	5 7	9 7	0 9	11 4
ATCC 10 953	11 59	0 43	3 8	6 5	0 7	10 6

The amino acid composition was studied qualitatively by paper and thin layer chromatography of 6N HCl hydrolysates From visual inspection of chromatograms the amino acid composition of the five preparations appeared to be very similar with the same 16–17 amino acids present in approximately the same proportional amounts

The results of the quantitative chemical analyses have been reported

in Table 1 The chemical composition of the five preparations was found to be essentially similar Cell walls from strain ATCC 10 953 appeared to contain slightly less polysaccharide than the other four preparations On the other hand the nitrogen values indicated that it contained more protein The quantitative data accounted for some 82–90 per cent of the materials examined without correction for water uptake during hydrolysis

In the test for 2 keto 3 deoxy sugar acids 2–3 mg samples of all cell wall preparations produced a faint purple colour with a small absorption maximum near 548 mμ Samples 0 5–1 0 mg of endotoxic lipopolysaccharide from *Fusobacterium* strain F1 (27) gave a distinct absorption maximum at this wavelength corresponding to the absorption maximum obtained with lipopolysaccharide from *Salmonella typhi* 0-901 (Difco) A small but distinct absorption maximum at 548 mμ was also obtained when the thiobarbituric acid test of *Warren* (45) was applied to 2 mg samples of the cell wall preparations The modified direct Ehrlich reaction (6) gave no absorption maximum at 530–565 mμ with 20 mg samples of cell walls from strains F1 and F2 Authentic samples 0 1–0 2 mg of N acetyl neuraminic acid produced a purple colour with absorption maximum at 530 mμ

TABLE 2

Quantitative Amino Acid Analysis of Undigested Cell Wall Preparation from Fusobacterium Strain F1

Amino acid	Moles per 100 moles total amino acids
Lysine	11 24
Histidine	1 16
Arginine	3 58
Aspartic acid	11 57
Threonine	4 68
Serine	4 62
Glutamic acid	12 99
Proline	5 01
Glycine	8 36
Alanine	10 08
Diaminopimelic acid	0 26
Valine	6 17
Methionine	1 84
Isoleucine	6 30
Leucine	8 14
Tyrosine	3 00
Phenylalanine	1 00

The spectra obtained in Warren s test gave no indication of the presence of 2 deoxyribose

A sample of the preparations from strain F1 was subjected to quantitative amino acid analysis The results are presented in Table 2 as moles per 100 moles total amino acids In addition to the amino acids

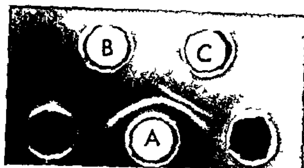


Fig 1

Precipitation lines in agar with cell fractions from *Fusobacterium* strain F1 Well A contained antiserum to microorganisms strain F1 Well B contained unconcentrated protoplasmic fraction and well C phenol water extract (water phase) from cell walls of the same strain Line 1 closest to well A

detected on chromatograms the presence of both leucine and *iso* leucine was demonstrated The presence of glucosamine and muramic acid was confirmed Among the amino acids lysine aspartic acid glutamic acid glycine alanine and leucine were present in relatively large amounts Small amounts of diaminopimelic acid were found

C *Presence of Major Precipitinogens in Strain F1 Cell Walls*

A suspension containing 10 mg of cell walls from strain F1 per ml of saline formed the same three lines in agar with homologous anti serum as did a buffer extract from crushed bacteria *viz* lines 1 2 and 3 (27) Line 2 was very weak and developed slowly However when antiserum to strain F1 had been absorbed with surplus amounts of lyophilized homologous phenol water extract prior to agar precipitation line 2 was more readily identified

Similar suspensions of cell walls F1 gave no definite precipitation lines in agar with heterologous antisera known to precipitate lines 4 and 5 When however such sera were absorbed for 1 hour at room temperature with 10 mg of cell walls F1 per ml of undiluted anti serum they were exhausted of precipitins against buffer extracts from crushed bacteria strain F1, as indicated by agar precipitation tests Similarly absorption of 1 ml antiserum to strain F1 with 10 mg of homologous cell walls completely removed precipitins responsible for lines 1 2 and 3 Two such absorptions were sometimes necessary in order to remove completely precipitins corresponding to line 2

Fifty mg of cell wall preparation from strain F1 were extracted by the phenol water method as described in (27) The water volume used was 5 ml The dialyzed water phase formed lines 1 and 3 with homo logous antiserum (Fig 1)

The protoplasmic fraction of strain F1 *i e* the supernatant obtained after the first centrifugation at 15 000 × *g* during cell wall prepara

tion was also tested for the presence of precipitinogens In these experiments extreme care was taken to avoid subjecting the bacteria to temperatures above 4 C after harvesting Undiluted protoplasmic material as well as a sample of the same material which had been concentrated ten times by freeze drying formed one line against homologous antisera The line showed deviation and complete fusion with line 1 (Fig 1) There was no evidence of spur formation even after several days The material giving this line could be recovered from the supernatant after the protoplasmic fraction had been precipitated with equal volumes of 0 5M trichloroacetic acid at 0 C and centrifuged as described for the native hapten of *E coli* by *Anacker et al* (3)

DISCUSSION

The chemical examinations showed that the cell wall preparations were free from significant amounts of contaminants containing nucleic acids Some preparations did contain visible amounts of a greyish material however Furthermore the electron micrographs of one preparation revealed the presence of small amounts of electron dense granular material The nature of these contaminants is not known In the literature on cell wall isolation procedures mention is frequently made of pigments and electron dense granules particularly with Gram negative bacteria (40 49) In most instances drastic chemical treatment or enzymatic digestion have to be resorted to in order to remove such materials For the purpose of the present study such procedures were not feasible since the cell walls were intended for serological as well as chemical investigations

Whether or to what extent envelope structures other than the cell wall proper was present in our preparations is not known

The overall chemical composition of the five preparations showed good agreement Strain ATCC 10 953 differed from the others in that the dominating hexose present in its cell wall was galactose rather than glucose Some variability between strains of fusobacteria in this respect is in agreement with the observations of *Baird Parker* (5) who found that either glucose or galactose or both were always present *Baird Parker* (5) reported that the presence of a high proportion of galactose was characteristic of strains classified by him as *Fusobacterium nucleatum* The type strain ATCC 10 953 is classified as a *Fusobacterium polymorphum* however It should be emphasized that doubt has been expressed as to the justification of maintaining a division between these two species within the genus *Fusobacterium* (7 33 39)

The cell and cell wall analyses of *Baird Parker* (5) further revealed an unidentified amino sugar ribose and two "slow moving components tentatively identified as muramic acid and diaminopimelic acid Small amounts of both of the two latter substances were demonstrated

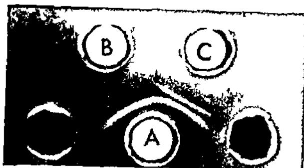


Fig 1

Precipitation lines in agar with cell fractions from *Fusobacterium* strain F1 Well A contained antiserum to microorganisms strain F1 Well B contained unconcentrated protoplasmic fraction and well C phenol water extract (*water phase*) from cell walls of the same strain Line 1 closest to well A

detected on chromatograms the presence of both leucine and *iso*leucine was demonstrated The presence of glucosamine and muramic acid was confirmed Among the amino acids lysine aspartic acid glutamic acid glycine alanine and leucine were present in relatively large amounts Small amounts of diaminopimelic acid were found

C Presence of Major Precipitinogens in Strain F1 Cell Walls

A suspension containing 10 mg of cell walls from strain F1 per ml of saline formed the same three lines in agar with homologous antiserum as did a buffer extract from crushed bacteria *viz* lines *1 2* and 3 (27) Line 2 was very weak and developed slowly However when antiserum to strain F1 had been absorbed with surplus amounts of lyophilized, homologous phenol water extract prior to agar precipitation line 2 was more readily identified

Similar suspensions of cell walls F1 gave no definite precipitation lines in agar with heterologous antisera known to precipitate lines 4 and 5 When however such sera were absorbed for 1 hour at room temperature with 10 mg of cell walls F1 per ml of undiluted antiserum they were exhausted of precipitins against buffer extracts from crushed bacteria strain F1 as indicated by agar precipitation tests Similarly absorption of 1 ml antiserum to strain F1 with 10 mg of homologous cell walls completely removed precipitins responsible for lines 1 2 and 3 Two such absorptions were sometimes necessary in order to remove completely precipitins corresponding to line 2

Fifty mg of cell wall preparation from strain F1 were extracted by the phenol water method as described in (27) The water volume used was 5 ml The dialyzed water phase formed lines 1 and 3 with homologous antiserum (Fig 1)

The protoplasmic fraction of strain F1 *i e* the supernatant obtained after the first centrifugation at 15 000 × *g* during cell wall prepara

tion was also tested for the presence of precipitinogens In these experiments extreme care was taken to avoid subjecting the bacteria to temperatures above 4 C after harvesting Undiluted protoplasmic material as well as a sample of the same material which had been concentrated ten times by freeze drying formed one line against homologous antisera The line showed deviation and complete fusion with line 1 (Fig 1) There was no evidence of spur formation even after several days The material giving this line could be recovered from the supernatant after the protoplasmic fraction had been precipitated with equal volumes of 0 5M trichloroacetic acid at 0 C and centrifuged as described for the native hapten of *E coli* by *Anacker et al* (3)

DISCUSSION

The chemical examinations showed that the cell wall preparations were free from significant amounts of contaminants containing nucleic acids Some preparations did contain visible amounts of a greyish material however Furthermore the electron micrographs of one preparation revealed the presence of small amounts of electron dense granular material The nature of these contaminants is not known In the literature on cell wall isolation procedures mention is frequently made of pigments and electron dense granules particularly with Gram negative bacteria (40 49) In most instances drastic chemical treatment or enzymatic digestion have to be resorted to in order to remove such materials For the purpose of the present study such procedures were not feasible since the cell walls were intended for serological as well as chemical investigations

Whether or to what extent envelope structures other than the cell wall proper was present in our preparations is not known

The overall chemical composition of the five preparations showed good agreement Strain ATCC 10 953 differed from the others in that the dominating hexose present in its cell wall was galactose rather than glucose Some variability between strains of fusobacteria in this respect is in agreement with the observations of *Baird Parker* (5) who found that either glucose or galactose or both were always present *Baird Parker* (5) reported that the presence of a high proportion of galactose was characteristic of strains classified by him as *Fusobacterium nucleatum* The type strain ATCC 10 953 is classified as a *Fusobacterium polymorphum* however It should be emphasized that doubt has been expressed as to the justification of maintaining a division between these two species within the genus *Fusobacterium* (7 33 39)

The cell and cell wall analyses of *Baird Parker* (5) further revealed an unidentified amino sugar ribose and two slow moving components" tentatively identified as muramic acid and diaminopimelic acid Small amounts of both of the two latter substances were demonstrated

in our preparations. Preliminary studies have indicated that they are constituents of a cell wall mucopeptide which has been purified by a procedure similar to that described by *Mandelstam* (30).

The sugar analyses of *Baird Parker* apparently were made on whole cell hydrolysates (5, 12). In our preparations no ribose was detected. The ribose found by *Baird Parker* (5) therefore probably did not originate from the cell wall.

The direct Ehrlich reaction gave no indication of the presence of sialic acids in our cell wall preparations. There is evidence that the coloured complex produced in the thiobarbituric acid reaction by 2-keto-3-deoxy sugar acids is identical to that produced by N-acetyl neuraminic acid (2, 45). Since the demonstration of 2-keto-3-deoxy octonate (KDO) in *Escherichia coli* 0–111 lipopolysaccharides by *Heath & Ghalambor* (21), KDO has been found in most lipopolysaccharides from Gram negative bacteria (29). Such a compound could not, however, be detected by *Hofstad* (23) in lipopolysaccharide preparations from *Bacteroides melaninogenicus*. Experiments aiming at the identification of the compound in cell walls from fusobacteria reacting in the thiobarbituric acid tests are in progress.

Heptoses are commonly found as constituents of endotoxic lipopolysaccharides of Gram negative bacteria (29). Since the heptose present in our preparations has not been identified, quantitative measurements have not been possible (13). Preliminary studies in which samples of four aldoheptoses with different molar extinction values in the cysteine sulphuric acid assay (kindly supplied by Dr. N. K. Richtmeyer, Bethesda, Md.) have been used as standards have indicated that heptose constitutes only a small part, probably less than 1 per cent, of our cell wall preparations.

As would be expected with these Gram negative bacteria, a relatively large number of amino acids was present in the cell wall preparations. The amino acids found by *Baird Parker* (5) to be dominating were also found in comparatively large amounts in our preparations, with the exception of phenylalanine, which was less predominant in our cell walls.

The method used for lipid measurements in the present study has been widely used for chemical analyses of bacterial lipopolysaccharides but only rarely for cell wall studies. As applied here, the method is reported to measure only esterified fatty acids (42). Information about possible interference from other substances has not been found. The method has the advantage of a high degree of sensitivity and has given very reproducible results in our laboratory. The values obtained can hardly be taken as measures of the total lipid content of the preparations, however.

As in other Gram negative bacteria, the endotoxic lipopolysaccharide and the accompanying acid hapten were found to be associated with the cell wall.

The demonstration of a precipitinogen in the protoplasmic fraction serologically related to the acid hapten may suggest the presence in fusobacteria of a "native hapten". Such substances have previously been demonstrated in several strains of *Escherichia coli* (4).

It has been shown that "acid hapten" from *Fusobacterium* strain F1 can be formed as a degradation product of the endotoxic lipopolysaccharide, e.g. on mild acid hydrolysis (27). The same relationship has been demonstrated with other Gram negative bacteria (37). The possibility that the precipitinogen found in protoplasm of *E. coli* and termed "native hapten" might be such a degradation product was excluded on basis of experimental evidence by *Anacker et al.* (3). The experimental conditions for the demonstration of the assumed "native hapten" in *Fusobacterium* strain F1 were similar to those employed by *Anacker et al.* (3). Although corroborative evidence will be needed, the most likely explanation of our findings to date is that a "native hapten" does occur in *Fusobacterium*.

The remaining three of the major precipitinogens found in strain F1 (27) also could be demonstrated to be present in the cell wall preparation either directly by agar precipitation against homologous antiserum or by absorption prior to agar precipitation of antisera known to precipitate the specific lines. The experiments indicated that considerable amounts of the materials responsible for these latter lines were removed from the cell wall fractions during the extensive washing procedures involved in the preparation of the cell walls.

SUMMARY

Undigested cell wall preparations from five strains of *Fusobacterium* contained polysaccharide, lipid and comparatively large amounts of protein. No contaminants containing nucleic acids were demonstrated.

Seventeen amino acids were identified. The cell walls also contained glucosamine, muramic acid, rhamnose, traces of xylose, a heptose, and glucose or galactose or both.

All of five major precipitinogens previously described in one of our strains were found to be present in undigested cell walls.

Evidence was found for the occurrence in *Fusobacterium* of "native haptens".

REFERENCES

1. *Acher, R., Fromageot, C. & Jutisz, M.*: Séparations chromatographiques d'acides aminés et de peptides. V. Les acides aminés individuels de l'insuline avec une note sur le dosage de la proline. Biochim. biophys. Acta (Amst.) 5, 81–88, 1950.
2. *Aminoff, O.*: Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochem. J. *81*, 384–392, 1961.
3. *Anacker, R. L., Finkelstein, R. A., Haskins, W. T., Landy, M., Milner, K. C., Ribi, E. & Stashak, P. W.*: Origin and properties of naturally occurring hapten from *Escherichia coli*. J. Bact. *88*, 1705–1720, 1964.

4 *Anacker R L Bickel W D Haskins W T Milner K C & Rudbach J A* Frequency of occurrence of native hapten among enterobacterial species J Bact 91 1427-1433 1966

5 *Baird Parker A C* The classification of fusobacteria from the human mouth. J gen Microbiol. 22 458-469 1960

6 *Barry G T Abbott V & Tsai T* Relationship of colominic acid (Poly N acetyl neuraminic acid) to bacteria which contain neuraminic acid J gen Microbiol 29 335-352 1962

7 *Boe J* *Fusobacterium* Studies on its bacteriology serology and pathogenicity Skr Norske Vidensk Akad I Mat nat Kl no 9 Oslo 1941 Pp 1-4-181

8 *Cifonelli J A & Smith F* Detection of glycosides and other carbohydrate compounds on paper chromatograms Analyt Chem 26 1132-1134 1954

9 *Consden R Gordon A H & Martin A J P* Qualitative analysis of proteins a partition chromatographic method using paper Biochem J 38 224-232 1944

10 *Cummins C S & Harris H* The chemical composition of the cell wall in some Gram positive bacteria and its possible value as a taxonomic character J gen Microbiol 14 583-600 1956

11 *Davies D A L* The identification of aldoheptose sugars Biochem J 67 253 256 1957

12 *Davis G H G & Baird Parker A C* Cell wall composition of *Leptotrichia* spp Nature (Lond) 183 1206 1207 1959

13 *Dische Z* Qualitative and quantitative colorimetric determination of heptoses J biol Chem 204 983-997 1953

14 *Dische Z* Color reactions of nuclei acid components In Chargaff E & Davidson J N (Eds) The nuclei acids vol 1 Academic Press New York 1955 pp 285-305

15 *Dische Z* New color reactions for determination of sugars in polysaccharides In Glick D (Ed) Methods of biochemical analysis vol 2 Interscience Publishers Inc New York 1955 pp 313-358

16 *Eastoe J F* Amino acid analysis of glycoproteins In Gottschalk A (Ed) Glycoproteins Elsevier Publishing Co Amsterdam 1966 p 112-150

17 *Edebo L* A new press for the disruption of microorganisms and other cells J biochem microbiol Technol Engng 2 453 479 1960

18 *Fiske C H & Subbarow Y* The colorimetric determination of phosphorus J biol Chem 66 375-400 1925

19 *Foster A B Horton D & Stacey M* Amino sugars and related compounds Part II Observations on the acidic hydrolysis of derivatives of 2 amino 2 deoxy D glucose (D glucosamine) J chem Soc 81-86 1957

20 *Grov A & Alsaker E* Amino acids in soil 1 Water soluble acids Acta chem scand 17 2307-2315 1963

21 *Heath E C & Ghalambor M A* 2 keto 3 deoxyoctonate a constituent of cell wall lipopolysaccharide preparations obtained from *Escherichia coli* Biochem biophys Res Commun 10 340-345 1963

22 *Hofstad T* An anaerobic oral filamentous organism possibly related to *Leptotrichia buccalis* 2 Composition of cell walls Acta path microbiol scand 70 461-468 1967

23 *Hofstad T* Chemical characteristics of *Bacteroides melaninogenicus* endotoxin Arch oral Biol 13 1149-1155 1968

24 *Jeanes A Wise C S & Dimler R J* Improved technique in paper chromatography of carbohydrates Analyt Chem 23 415-420 1951

25 *Kabat E A & Mayer M M* Experimental immunochemistry Thomas Springfield II ed 1961 pp 476-483

26 *Kauffmann F Lüderitz O Stierlin H & Westphal O* Zur Immunchemie der O Antigene von *Enterobacteriaceae* I Analyse der Zuckerbausteine von *Salmonella* O Antigenen Zbl Bakt I Abt Orig 178 44-458 1960

27 *Kristoffersen T* Immunochemical studies on oral fusobacteria I Major precipitinogens Acta path microbiol scand 77 235-246 1969

28 *Linskens H F* Papierchromatographie in der Botanik Springer Verlag Berlin 1955 p 164

29 *Lüderitz O Staub A M & Westphal O* Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae* Bact Rev 30 192 255 1966

30 *Mandelstam J* Preparation and properties of the mucopeptides of cell walls of gram negative bacteria Biochem J *84* 294–299 1962
31 *Merck E* Chromatographie E. Merck A/G Darmstadt p 135
32 *Moore S Spackman D H & Stein W H* Chromatography of amino acids on sulfonated polystyrene resins An improved system Analyt Chem *30* 1185–1190 1958
33 *Omata R R & Braunberg R C* Oral Fusobacteria J Bact *80* 737–740 1960
34 *Osborn M J* Studies on the Gram negative cell wall I Evidence for the role of 2 keto 3 deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc Nat Acad Sci (Wash) *50* 499–506 1963
35 *Partridge S M* Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars Nature (Lond) *164* 443 1949
36 *Reed L J* The occurrence of γ aminobutyric acid in yeast extracts its isolation and identification J biol Chem. *183* 451–458 1950
37 *Ribi E. Haskins W T Milner K C Anacker R L Ritter D B Goode G Trapani R J & Landy M* Physicochemical changes in endotoxin associated with loss of biological potency J Bact *84* 803–814 1962
38 *Rondle C J M & Morgan W T J* The determination of glucosamine and galactosamine Biochem J *61* 586–589 1955
39 *Rosebury T* Microorganisms indigenous to man McGraw Hill Book Co Ltd New York 1962 Pp 144–185
40 *Salton M R J* The bacterial cell wall Elsevier Publishing Co Amsterdam 1964 P 62
41 *Smith I* Chromatographic and electrophoretic techniques Vol I Chromatography Interscience Publishers Inc New York 1960 Pp 252–253
42 *Snyder F & Stephens N* A simplified spectrophotometric determination of ester groups in lipids Biochim. biophys Acta (Amst) *34* 244–245 1959
43 *Spackman D H Stein W H & Moore S* Automatic recording apparatus for use in the chromatography of amino acids Analyt Chem *30* 1190–1206 1958
44 *Trevelyan W E Procter D P & Harrison J A* Detection of sugars on paper chromatograms Nature (Lond) *166* 444–445 1950
45 *Warren L* The thiobarbituric acid assay of sialic acids J biol Chem *234* 1971–1975 1959
46 *Weissbach A & Hurwitz J* The formation of 2 keto 3 deoxyheptonic acid in extracts of *Escherichia coli* B I Identification J biol Chem *234* 705–709 1959
47 *White B N Shetlar M R Shurley H M & Shilling J A* Incorporation of D ($1^{14}C$) galactosamine into serum proteins and tissues of the rat Biochim biophys Acta (Amst) *101* 259–266 1965
48 *Winzler R J* Determination of serum glycoproteins In Glick D (Ed) *Methods of biochemical analysis* vol 2 Interscience Publishers Inc New York 1955 p 279–311
49 *Work E* Biochemistry of the bacterial cell wall Nature (Lond) *179* 841–847 1957
50 *Yoshida A Heden C G Cedergren B & Edebo L* A method for the preparation of undigested bacterial cell walls J biochem microbiol Technol Engng *3* 151–159 1961
51 *Youngburg G E & Youngburg M V* Phosphorus metabolism I A system of blood phosphorus analysis J Lab Clin Med *16* 158–166 1930

Acta path microbiol scand 77 258–266 1969

Institute for Surgical Research University Hospital Rikshospitalet
Oslo Norway

OPSONIZING AND ^{32}P RELEASING ACTIVITIES OF RAT INTESTINAL STRANGULATION FLUID ON ^{32}P LABELLED *E COLI*

By

ARNE TRIPPESTAD

Received 26 iii 69

The peritoneal fluid of intestinal strangulation obstruction is lethal when injected into the peritoneal cavity of healthy animals in small quantities (5 16)

Similar quantities of sterile filtrates of *rat or canine peritoneal strangulation* fluid are usually nonlethal to recipient animals (3 5 15 20) while lethality is retained in sterile filtrates of rabbit *strangulation* fluid presumably due to bacterial exotoxins (4 6) The importance of bacterial factors is further stressed by the fact that strangulation fluid collected from germfree rats is nonlethal to assay mice (2 9)

In nonlethal sterile filtrates of rat strangulation fluid the lethal effect can be re established by suspending viable *E coli* into the filtrates (3 7) The number of *E coli* required to kill assay mice is however smaller when the bacteria are suspended into filtrates of lethal strangulation fluid than when suspended into normal saline (1 7) rat plasma (1) or human serum (7) Such enhanced virulence of *E coli* is apparent only when the bacteria are suspended in filtrates of rat strangulation fluid collected during the later stages of the ailment (3) The enhanced virulence is probably not due to bacterial products since *Amundsen* (1) has shown that filtrates of strangulation *fluid from* germfree rats also enhances the virulence of *E coli*

Enhanced bacterial virulence may result from interference with normal antibacterial defence mechanisms

The bactericidal effect *in vitro* of mammalian sera on *E coli* and other Gram negative bacteria is well known This reaction in which antibodies and complement components are involved is believed to

---

The author is greatly indebted to *T Mitlvedt* MD of Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt who has carried out *the* culturing and quantitation of the bacteria and to mr *J Haugersteen* who has given valuable advice in statistics

This investigation was supported by grants from *Norges Almenvitenskapelige Forskningsråd Norsk Forening til Kreftens Bekjempelse* and Norsk *Medisinaldepot*

represent one aspect of the antibacterial defence system (13) During incubation of ³²P labelled *E. coli* suspended in media containing fresh serum labelled substances are released from the bacteria into the medium in proportion to the number of bacteria being killed (18 19) In the present study the release of label into the medium has been utilized as an indirect measure of the bactericidal effect of the medium used

The major role of the phagocytic cells in the antibacterial defence is generally accepted *E coli* are poorly phagocytized unless the bacteria have been opsonized by serum components similar to but not necessarily identical with the components involved in the humoral bactericidal reaction In this study phagocytosis was estimated indirectly by measuring the incorporation of radioactivity by rat polymorphonuclear leucocytes incubated in suspensions of ³²P labelled *E coli*

The aim of the present study has been to investigate the influence of filtrates of rat strangulation fluid on phagocytosis and also study the humoral bactericidal capacity of the filtrates on the strain of *E coli* tested

MATERIALS AND METHODS

Reagents

Sodium iodoacetate (IAA Fluka AG Buchs SG Switzerland) was dissolved in Krebs Ringer phosphate buffer containing 10^{-2} M glucose (KRG (17)) The final concentration of 10^{-3} M IAA in the medium was obtained by diluting a freshly prepared stem solution of 10^{-2} M

Other reagents were purchased from standard commercial sources

Strangulation Fluid

Strangulation fluid was collected from rats as described by *Amundsen & Midtvedt* (3) 100 male rats of a local strain were used weighing between 175 and 310 g The fluid was collected at 4 C over 24 hour periods Each portion was immediately centrifuged at 50 000 × g 60 min/0 C to remove cells and bacteria The supernatant was stored at —20 C The portions collected during the first 24 hour period were pooled and are referred to as the "early" strangulation fluid in the following Similarly a pool of "late" strangulation fluid was prepared from portions collected later than 48 hours Each pool of fluid was centrifuged at 100 000 × g/60 min/0 C in a Spinco model L 5 ultracentrifuge The supernatant was passed through a Millipore filter with pore size 0 22 μ The filtrate was tested for sterility and stored at —20 C

Sterility Test

Aliquots of 0 1 ml of the filtrates were transferred to glucose broth and Todd Hewitt broth (Oxoid) and incubated aerobically and anaerobically at 37 C Anaerobic conditions were provided by means of the pyrogallol method (11) The filtrates were considered sterile if no growth occurred at the end of an incubation period of 72 hours

Bacteria

The strain of *E coli* used was the same as the one used in experiments reported previously where the techniques of culture and labelling with ³²P were also described (19)

Serum

Pooled rat serum was used in all experiments (19)

Polymorphonuclear Leucocytes (PMN)

were aspirated from the peritoneal cavity of rats of either sex following intra peritoneal injection of 1% per cent sodium caseinate Monolayers of PMN were pre pared in tissue culture tubes (19)

Phagocytosis

The technique for determination of phagocytosis has been described in detail elsewhere (19) In each experiment quadruplets of tissue culture tubes containing monolayers of PMN were incubated at 37 C with ³ P labelled *E coli* suspended into the medium to be tested (10⁹ per ml) After an incubation period of 15 min the incorporation of radioactivity and the protein content of the cells layer were determined Phagocytosis was expressed as cpm per mg cell protein

Release of Label into the Medium

Aliquots of a suspension of ³ P labelled *E coli* (10⁹ per ml) in the medium to be tested were incubated at 37 C in quadruplets of tissue culture tubes Following an incubation period of 15 min the suspension was cooled in ice water and centrifuged at 6000 × g/10 min/0 C The release of label expressed as cpm per ml of medium was determined in aliquots of the supernatant as previously described (19)

Statistical Analysis

was carried out by means of the two sample ranks test of Wilcoxon White (12) Results from different experiments were compared by converting the absolute values into percentages of the control i e the mean value obtained in the control medium of the same experiment

RESULTS

Phagocytosis

Fig 1 illustrates the rate of incorporation into PMN of ³ P labelled *E coli* suspended into media of different composition When the bac

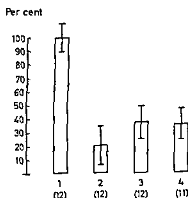


Fig 1

Phagocytosis of ³ P labelled *E coli* by PMN during incubation in media of different composition

Aliquots (2 5 ml) of a suspension of ³ P labelled *E coli* (10⁹ per ml) in the medium to be tested were incubated at 37 C for 15 min in quadruplets of tissue culture tubes containing monolayers of PMN The media consisted of 1) KRG with 5 per cent serum (control) 2) KRG 3) KRG with 10 per cent early strangulation fluid filtrate and 4) KRG with 10 per cent late filtrate Each column represents the mean of individual observations in three experiments ± standard deviation expressed as percentage of the controls The numbers in brackets indicate the total number of observations

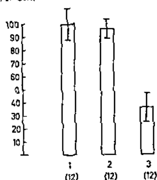


Fig 2

Phagocytosis of ³²P labelled *E coli* by PMN preincubated in late strangulation fluid filtrate or IAA

Quadruplets of monolayers of PMN were pre incubated for 30 min at 37 C in different media which consisted of 1) KRG 2) KRG with 10 per cent late strangulation fluid filtrate and 3) KRG with 10⁻³ M IAA The preincubation medium was removed and the cell layer washed once in KRG Aliquots of 0 5 ml of a suspension of ³²P labelled *E coli* (10⁹ per ml) in KRG containing 5 per cent serum was added to each tube Following a second incubation period of 15 min at 37 C the incorporation of labelled bacteria into the PMN was determined Each column illustrates the mean of individual observations in three experiments ± standard deviation expressed as percentage of the controls The numbers in brackets indicate the total number of observations

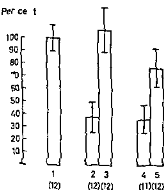


Fig 3

Phagocytosis of ³²P labelled *E coli* by PMN in media containing strangulation fluid filtrates and serum

Aliquots of 0 5 ml of a suspension of ³²P labelled *E coli* (10⁹ per ml) in the medium to be tested were incubated at 37 C for 15 min in quadruplets of tissue culture tubes containing monolayers of PMN The media consisted of 1) KRG with 5 per cent serum (control) 2) KRG with 10 per cent early filtrate 3) KRG with 10 per cent early filtrate and 5 per cent serum 4) KRG with 10 per cent late filtrate and 5) KRG with 10 per cent late filtrate and 5 per cent serum Each column represents the mean of individual observations in three experiments ± standard deviation expressed as percentage of the controls The numbers in brackets indicate the total number of observations

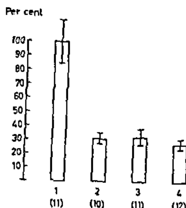


Fig 4

Release of ^{32}P from labelled *E coli* incubated in various media
Aliquots (2 5 ml) of a suspension of ^{32}P labelled *E coli* (10^9 per ml) in the medium to be tested were incubated at 37 C for 15 min in quadruplets The media consisted of 1) KRG with 5 per cent serum (control) 2) KRG 3) KRG with 10 per cent early strangulation fluid filtrate and 4) KRG with 10 per cent late strangulation fluid filtrate The columns represent the mean of individual observations in three experiments ± standard deviation expressed as percentage of the controls The numbers in brackets indicate the total number of observations

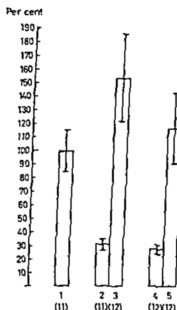


Fig 5

Release of ^{32}P from labelled *E coli* incubated in media containing *strangulation* fluid filtrates and serum

Aliquots (2 5 ml) of a suspension of ^{32}P labelled *E coli* (10^9 per ml) in the medium *to be tested were incubated in quadruplets at 37 C for 15 min* The media consisted of 1) KRG with 5 per cent serum (control) 2) KRG with *10 per cent "early"* strangulation fluid filtrate 3) KRG with 10 per cent *early filtrate* and 5 per cent serum 4) KRG with 10 per cent late filtrate and 5) KRG *with* 10 per cent "late" filtrate and 5 per cent serum *Each column represents the mean of individual* observations in three experiments ± *standard deviation* expressed as percentage of the controls The numbers in brackets indicate the total number of observation

teria were suspended into KRG alone the incorporation of labelled bacteria was small A slightly larger incorporation occurred when the suspending medium contained 10 per cent of either filtrate in KRG ($P < 0.05$) The uptake of labelled *E coli* in these media in which serum was absent was less than 40 per cent of that observed in KRG containing 5 per cent serum The rate of incorporation of labelled bacteria in KRG with 10 per cent early filtrate was not different from that found in KRG containing 10 per cent of the late filtrate ($p > 0.10$)

The effect of preincubation of the PMN in different media will be seen from Fig 2 Experimental details are described in the figure text Pre incubation of the PMN in IAA reduced the average uptake of labelled *E coli* to 35 per cent of the controls The rate of uptake by the PMN preincubated in the late filtrate was not significantly different from that of the controls ($p > 0.10$)

When serum was added to suspensions of ³²P labelled *E coli* in KRG with 10 per cent of the late filtrate the uptake of labelled bacteria was substantially larger than that observed in the same medium in the absence of serum (Fig 3) The degree of incorporation was however significantly smaller than that obtained when the bacteria were suspended in KRG with 5 per cent serum ($p < 0.01$) When serum was added to labelled *E coli* suspended in KRG with 10 per cent of the early filtrate the degree of incorporation was not different from that observed in KRG with 5 per cent serum ($p > 0.10$)

Release of ³²P into the medium

From Fig 4 it can be seen that the release of label into the medium following incubation of the bacterial suspension at 37 C was small when the suspending medium contained KRG alone or KRG with 10 per cent of either early or late filtrate The release of label observed in KRG containing 10 per cent of either filtrate was not different from the release found in KRG alone ($p > 0.10$) In the absence of serum the release of ³²P was only 30 per cent of that seen in the control medium which contained 5 per cent serum in KRG

Following the addition of 5 per cent serum to suspensions of *E coli* in KRG containing 10 per cent of either early or late filtrate the release of label was substantially larger than the release found in the corresponding media in the absence of serum (Fig 5) When serum was added to the medium containing the early filtrate the release of label was considerably larger than the release obtained in the control medium which contained 5 per cent serum in KRG ($p < 0.01$) When the same amount of serum was added to media containing the late filtrate the release of ³²P was not significantly different from the amount of label released into the control medium ($p > 0.10$)

DISCUSSION

The results of the present study show that phagocytosis of ^{32}P labelled *E coli* was substantially smaller in KRG containing filtrates of strangulation fluid than in KRG with serum This might be due to inhibition of the phagocytotic function of the PMN or insufficient opsonization of the bacteria

The strangulation fluid filtrates did not appear to affect the phagocytotic function of the PMN directly since pre incubation of the PMN in the late filtrate did not reduce the uptake of labelled *E coli* In contrast a substantial reduction of phagocytosis was observed following pre incubation of the PMN in IAA This substance is known to inhibit the phagocytotic activity of PMN (8) It seems likely that the small degree of phagocytosis observed when the media contained filtrates of strangulation fluid might be due to deficient opsonization of the bacteria

Addition of serum to media containing filtrates of strangulation fluid might be expected to increase the rate of phagocytosis to the level seen in media containing the same amount of serum alone by supplying the necessary opsonins This was in fact accomplished when serum was added to the early filtrate Addition of serum to the late filtrate also increased phagocytosis but the level obtained was significantly smaller than that found in the control medium which contained the same amount of serum A reduced incorporation of label might be produced by the decrease in specific activity of the bacteria which occurs when labelled substances are released into the medium (19) Since the release of ^{32}P was not significantly larger than that seen in the control medium the presence of inhibitory substances in the late filtrate seemed possible capable of interfering with the opsonic activity of serum This problem will be considered in a separate report

It has been demonstrated that the release of label into the medium from ^{32}P labelled *E coli* which occurs during incubation in media containing serum reflects the bactericidal activity of the serum used (18 19) When labelled bacteria were incubated in KRG containing filtrate of strangulation fluid the release of ^{32}P was not larger than that found in KRG alone This finding indicates that the bactericidal activity of either filtrate was insignificant Accordingly serum components involved in opsonization as well as humoral destruction of *E coli* appeared to be absent or inactivated in the filtrates

The addition of serum to media containing the filtrates produced an increased release of ^{32}P into the medium When serum was added to the early filtrate the release was substantially larger than the release observed in control media containing the same amount of serum This indicates the presence of substances in the early filtrate which enhance the ^{32}P releasing activity of serum yet being unable to initiate the reaction by itself

Humoral components participating in the bactericidal and opsonic processes might be expected to appear in filtrates of strangulation fluid if the latter is derived from serum Lack of humoral components in the filtrates might result from depletion of such components by the bacteria originally present in the strangulation fluid or by bacterial products Large numbers of bacteria can be cultivated from strangulation fluid collected from rats during the late course of the ailment while specimens obtained during the first 24 hour period are usually sterile (3)

Substances of non bacterial origin might also be responsible for such depletion Complement activation (10) with generation of chemotactic substances and fixation of antibody and complement components (13) has been demonstrated in vitro after incubation of various subcellular substances in normal homologous sera It seems reasonable to assume that substances with antigenic properties may be released from the necrotic bowel

The findings presented in this report do not explain the virulence enhancing effect of filtrates of late strangulation fluid on *E coli* (3) since the opsonizing and the ^{32}P releasing capacities of the late filtrate was not significantly different from those of the early filtrate

The small antibacterial effects demonstrated in media containing filtrates of strangulation fluid might be of importance in the pathophysiology of intestinal strangulation obstruction by creating conditions within the peritoneal cavity favorable for bacterial growth

SUMMARY

The influence of sterile filtrates of rat strangulation fluid on phagocytosis of ^{32}P labelled *E coli* was studied *in vitro* The rate of phagocytosis by rat polymorphonuclear leucocytes was small in media containing the filtrates The filtrates did not seem to interfere directly with the function of the phagocytes Lack of opsonizing activity in the filtrates was suggested This was supported by the finding of a small release of label from the bacteria during incubation of ^{32}P labelled *E coli* suspended in the filtrates in contrast to the large release of ^{32}P observed when the suspending medium contained rat serum

REFERENCES

1 *Amundsen E* Studies on toxicity enhancing factor in fluid from experimentally strangulated intestinal loop in the rat J surg Res *4* 531–536 1964

2 *Amundsen E & Gustafsson B E* Results of experimental intestinal strangulation obstruction in germ free rats J exp Med *117* 823 839 1963

3 *Amundsen E & Mellbye O J* The toxicity of fluid from experimentally strangulated intestinal loops in the rat J surg Res *4* 306–313 1964

4 *Bajaj I S Sachdeva H S & Anand S S* Experimental strangulation obstruction Evaluation of the toxicity factor with special reference to in vivo blood changes J surg Res *7* 254–260 1967

5 *Barnett W O* Experimental strangulated intestinal obstruction A review Gastroenterology *39* 34–40 1960

6 *Bornside G H & Cohn I jr* Clostridial toxins in strangulation intestinal ob struction in the rabbit Surgery *152* 330–342 1960
7 *Bornside G H Kuebler W J & Cohn I jr* Enhanced bacterial virulence i fluids produced in strangulation intestinal obstruction Proc Soc exp Biol (N Y) *121* 551–555 1966
8 *Brumfitt W Glynn A A & Percival A* Factors influencing the phagocytosi of *Escherichia coli* Brit J exp Path *46* 215–226 1965
9 *Cohn I jr Floyd C E Dresden C F & Bornside C H* Strangulation obstruc tion in germfree animals Ann Surg *156* 692–701 1962
10 *Elson C J & Weir D M* Chemotaxis of polymorphs induced by tissue antigen and normal serum in rats a possible clearance mechanism Clin exp Im munol 2 581–588 1967
11 *Gustafsson B E Midtvedt T & Norman A* Isolated fecal microorganism capable of 7 α dehydroxylating bile acids J exp Med *123* 413–432 1966
12 *Mainland D* Elementary medical statistics 2 ed W B Saunders Company Philadelphia and London 1964
13 *Muschel L H* Serum bactericidal actions Ann N Y Acad Sci *88* 1265–1272 1960
14 *Muschel L H Ahl L A & Hong R* Complement fixation with tissue antigens Immunochemistry *4* 501–503 1967
15 *Nagy I & Weipers W I* A study of bacteria and lethal factors in fluids from experimental intestinal obstruction in dogs J Path Bact 95 199 210 1968
16 *Nemir P Hawthorne H R Cohn I jr & Drabkin D L* The cause of death in strangulation obstruction An experimental study II Lethal action of the peritoneal fluid Ann Surg *130* 874–880 1949
17 *Roberts J & Quastel J H* Particle uptake by polymorphonuclear leucocytes and Ehrlich ascites carcinoma cells Biochem J *89* 150–156 1963
18 *Spitznagel J K & Wilson L A* Normal serum cytotoxicity for P^{32} labeled smooth Enterobacteriaceae I Loss of label death and ultrastructural damage J Bact *91* 393–400 1966
19 *Trippestad A & Midtvedt T* Phagocytosis of ^{32}P labelled E coli by rat poly morphonuclear leucocytes *Evaluation of a method* Acta path microbiol scand *74* 259–273 1968
20 *Turner M D Grogan J B & Truett G W* A study of the lethal mechanism in experimental closed loop strangulated obstruction of the small intestine Amer J Surg *102* 560–567 1961

Acta path microbiol scand 77 267–274 1969

Institute for Surgical Research University Hospital Rikshospitalet Oslo Norway

INFLUENCE OF RAT INTESTINAL STRANGULATION FLUID ON THE OPSONIC AND ^{32}P RELEASING ACTIVITY OF SERUM ON ^{32}P LABELLED *E COLI*

By

ARNE TRIPPESTAD

Received 18 iv 69

Sterile filtrates of exudate accumulating in the peritoneal cavity during the late course of intestinal strangulation obstruction have a virulence enhancing effect on *E coli* suspended in the filtrates (1 3) Filtrates of strangulation fluid collected during the early course of the ailment do not possess such properties The mechanism(s) causing enhanced virulence is unknown Interference with body defence mechanisms by substances in the filtrates might be involved

In a previous study (9) it was reported that filtrates of strangulation fluid from rats were unable to stimulate phagocytosis of ^{32}P labelled *E coli* by rat polymorphonuclear leucocytes A toxic effect on the phagocytes by substances in the filtrates could not be demonstrated The absence of opsonizing components in the filtrates seemed likely Furthermore it appeared that the filtrates collected during the late course of strangulation obstruction also might contain substances which were capable of interfering with the opsonic system of serum

The aim of the present study was to investigate the influence of strangulation fluid filtrates on serum components involved in antibacterial defence An *in vitro* system based on radioisotope technique has been used (10)

MATERIALS AND METHODS

Strangulation Fluid

was collected from rats as described by *Amundsen & Midtvedt* (9) The samples used for comparative studies were the same as those employed in previously reported experiments (9) Fluid pooled from other rats was used in the time course studies The pooled filtrates were prepared and tested for sterility as previously described (9)

The author is greatly indebted to *T Midtvedt* M D of Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt, who was carried out the culturing and quantitation of the bacteria and to mr *J Haugersveen* who has given valuable advice in statistics

This investigation was supported by grants from *Norges Almenvitenskapelige Forskningsråd Norsk Forening til Kreftens Bekjempelse* and *Norsk Medisinaldepot*

Bacteria

The strain of *E coli* used was the same as the one employed in earlier studies (10)

Serum and Polymorphonuclear Leucocytes (PMN)

were pooled from rats of the same strain as the one used for collection of strangulation fluid as described elsewhere (10)

Preincubation of Media and Preparation of Test Suspensions

In some experiments a final concentration of 10 per cent serum in the medium was used while in the majority of experiments the serum was diluted with equal parts of Krebs Ringer buffer containing 10 mM of glucose (KRG (6)) before being added to the medium to make a final concentration of 5 per cent serum

One part of the filtrate of strangulation fluid seven parts of KRG and one part of serum (or serum dilution) were mixed in that sequence

Control media were prepared by mixing eight parts of KRG with one part of serum (or serum dilution)

Each test medium was prepared in two glass tubes One of the tubes was preincubated at 37 C in a water bath for 60 min The other tube was placed in ice water for an equal period of time

At the end of this period one part of a suspension of ^{32}P labelled *E coli* (10^{10} per ml) was mixed with nine parts of the medium to be tested

When not incubated the media and test suspensions were always kept at 0 C All test suspensions contained 5 or 10 per cent serum and 10^9 ^{32}P labelled *E coli* per ml

Determination of Phagocytosis and of ^{32}P Released into the Medium

The techniques used have been described in another report (10) Details of the various experiments are described in the figure texts

Statistical Analysis

was carried out by means of the two sample ranks test of Wilcoxon White (4 9)

RESULTS

Phagocytosis

In Fig 1 the incorporation of ^{32}P labelled *E coli* by PMN is illustrated following preincubation of various media at 0 C and 37 C

The control medium contained 5 per cent serum in KRG When the control medium was preincubated at 37° C the subsequent incorporation of labelled *E coli* into the PMN was not different from that observed when the control medium was preincubated at 0 C ($p > 0.10$)

In a mixture of 10 per cent of the "early" strangulation fluid filtrate and 5 per cent serum in KRG the ingestion of labelled *E coli* by the PMN was not significantly different from that found in the control medium when both media had been preincubated at 0 C prior to the addition of labelled bacteria When the mixture of "early" filtrate and serum was preincubated at 37 C the subsequent ingestion of labelled *E coli* by the PMN was 17 per cent less than when the control medium was preincubated at the same temperature ($p < 0.05$)

In a mixture containing 10 per cent "late" strangulation fluid filtrate and 5 per cent serum in KRG the ingestion of labelled *E coli* by the PMN was 27 per cent smaller than that found in the control medium

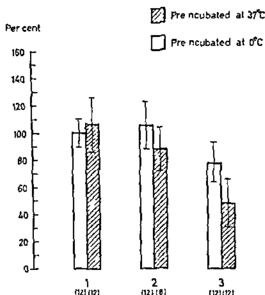


Fig 1

Phagocytosis of ^{32}P labelled *E coli* by PMN in media containing strangulation fluid filtrates and serum preincubated at 0 and 37 C. The media were preincubated as described in Materials and Methods prior to the addition of ^{32}P labelled *E coli*. Aliquots (2.5 ml) of a suspension of labelled *E coli* (10^9 per ml) in the medium to be tested were incubated at 37 C for 15 min in quadruplets of tissue culture tubes containing monolayers of PMN. The media consisted of 1) KRG with 5 per cent serum (control) 2) 10 per cent "early" strangulation fluid filtrate and 5 per cent serum in KRG and 3) 10 per cent "late" strangulation fluid filtrate and 5 per cent serum in KRG. Each column represents the mean of individual observations in three experiments ± standard deviation expressed as percentages of the controls. The numbers in brackets indicate the total number of observations.

when both media were preincubated at 0 C ($p < 0.01$). When the same mixture was preincubated at 37 C the uptake of labelled bacteria by the PMN was about 40 per cent smaller than the uptake when the mixture was preincubated at 0 C. In a mixture of "late" filtrate and serum which had been preincubated at 37 C the incorporation of labelled bacteria into the PMN was about 45 per cent smaller than that observed when the medium contained a similarly preincubated mixture of "early" strangulation fluid filtrate and serum.

Fig 2 illustrates a typical time course experiment in which the period of contact between labelled *E coli* and PMN varied. In a medium containing KRG with 10 per cent of the "late" filtrate and 10 per cent serum preincubated at 37 C the uptake of labelled *E coli* by the PMN was small during the first 15 min period of incubation. Further prolongation of the incubation period caused a marked increase in the rate of incorporation.

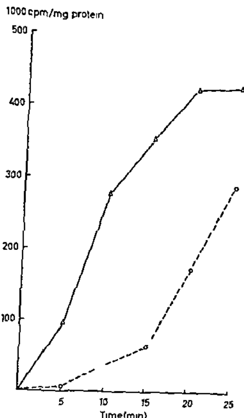


Fig 2

Phagocytosis of ³ P labelled *E coli* by PMN during various periods of incubation following preincubation of late strangulation fluid filtrate and serum at 37 C. Aliquots (2 5 ml) of a suspension of ³ P labelled *E coli* (10^9 per ml) were incubated at 37 C with monolayers of PMN Single tubes were used f r each period of incubation Incorporation of labelled *E coli* by the PMN was expressed as cpm per mg of cell protein o —— o Phagocytosis when the medium contained 10 per cent *late strangulation fluid filtrate and 10 per cent serum in KRG preincubated at* 37 C for 60 min prior to the addition of labelled bacteria △—— △ Phagocytosis when the medium contained 10 per cent serum in KRG preincubated at 37 C for 60 min The late filtrate of this experiment was prepared fr m specimens collected between 24 and 48 hours after induction of intestinal strangulation obstruction in 33 rats of both sexes

Release of Label into the Medium

Fig 3 shows the release of ³ P from labelled *E coli* during incubation at 37 C when the media had been preincubated at 0 C or at 37 C prior to the addition of labelled bacteria

The control medium contained 5 per cent serum in KRG When the bacteria were suspended in control medium preincubated at 37 C the subsequent release of ³ P into the medium was not significantly different from the release observed in control medium preincubated at 0 C ($p > 0\ 05$)

In a mixture of 10 per cent early strangulation fluid filtrate and

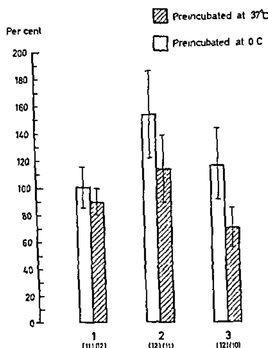


Fig 3

Release of ³²P from labelled *E coli* in media containing strangulation fluid filtrates and serum preincubated at 0 and 37 C The media were preincubated as described in Materials and Methods prior to the addition of ³²P labelled *E coli* Aliquots (2 5 ml) of a suspension of labelled *E coli* (10^9 per ml) in the medium to be tested were incubated in quadruplets at 37 C for 15 min The media consisted of 1) KRG with 5 per cent serum (control) 2) 10 per cent early strangulation fluid filtrate and 5 per cent serum in KRG and 3) 10 per cent late strangulation fluid filtrate and 5 per cent serum in KRG Each column represents the mean of individual observations in three experiments ± standard deviation expressed as percentages of the controls The numbers in brackets indicate the total number of observations

5 per cent serum in KRG the release of label was more than 50 per cent higher than that found in the control medium when both media were preincubated at 0 C

When the mixture of early filtrate and serum was preincubated at 37 C the release of label was less than when the same medium was preincubated at 0 C ($p < 0\ 01$) The release observed when the mixture of early filtrate and serum was preincubated at 37 C was however larger than the release found when the bacteria were suspended in control medium preincubated at 37 C ($p < 0\ 05$)

In a medium containing 10 per cent late filtrate and 5 per cent serum in KRG the release of ³²P was not significantly different from the release found in control medium when both media were preincubated at 0 C ($p > 0\ 05$) Following preincubation of the mixture of late filtrate and serum at 37 C the release of label was almost 60

reduced during prolonged incubation with the labelled *E coli* The partly reversed inhibition was not observed when filtrates with more potent activities were used

It seems reasonable to assume that substances in the late filtrate might interfere with components of either antibody or complement character since both groups of serum components are believed to participate in the opsonization of *E coli* (7) as well as in the humoral destruction of the same bacteria (5)

It seems likely that the inhibitory effects of the "late strangulation fluid on serum components may play a part in the pathophysiology of intestinal strangulation obstruction Interference with such components might favour local proliferation of bacteria in the peritoneal cavity and perhaps also spreading of bacteria to other organs One cannot however from these results conclude that the observed effects are responsible for the virulence enhancing property of the "late strangulation fluid filtrate

SUMMARY

Phagocytosis of ³ P labelled *E coli* by rat PMN was determined *in vitro* Release of ^{32}P into the medium from the labelled bacteria was also determined Sterile filtrates of exudates from a strangulated loop of rat intestine appeared to inhibit the opsonic and ³ P releasing activity of rat serum when preincubated with serum at 37 C The inhibitory principles were present in filtrates of strangulation fluid collected during the late course of intestinal strangulation obstruction but were not detected in fluid collected during the early course of the ailment The findings suggest that the opsonic as well as the humoral bactericidal systems might be impaired during intestinal strangulation obstruction

REFERENCES

1 *Amundsen E* Studies on a toxicity enhancing factor in fluid from experimentally strangulated intestinal loops in the rat J surg Res 4 531–536 1964
2 *Amundsen E & Midtvedt T* The toxicity of fluid from experimentally strangulated intestinal loops in the rat J surg Res 4 306–313 1964
3 *Bornside G H Kuebler W J & Cohn I jr* Enhanced bacterial virulence in fluids produced in strangulation intestinal obstruction Proc Soc exp Biol (N Y) *121* 551–555 1966
4 *Mainland D* Elementary medical statistics 2 ed W B Saunders Company Philadelphia & London 1964 p 283
5 *Muschel L H* Serum bactericidal actions Ann N Y Acad Sci *88* 1265–1272 1960
6 *Roberts J & Quastel J H* Particle uptake by polymorphonuclear leucocytes and ascites carcinoma cells Biochem J *89* 150–156 1963
7 *Rowley D* Phagocytosis and immunity Experientia *22* 1–13 1966
8 *Spitznagel J K & Wilson I A* Normal serum cytotoxicity for P³² labeled smooth *Enterobacteriaceae* I Loss of label death and ultrastructural damage J Bact *91* 39 –400 1966
9 *Trippestad A* Opsonizing and ³ P releasing activities of rat intestinal strangulation fluid on ³ P labelled *E coli* Acta path microbiol scand *77* 258–266 1969
10 *Trippestad A & Midtvedt T* Phagocytosis of ³²P labelled *E coli* by rat polymorphonuclear leucocytes Evaluation of a method Acta path microbiol. scand *74* 259–273 1968

Acta path microbiol scand 77 275–287 1969

Department of Biology University of Strathclyde Glasgow C1 Scotland and Department of Toxoplasmosis and Viral Diseases Statens Serum institut Copenhagen Denmark

OBSERVATIONS ON THE FAECAL TRANSMISSION OF *TOXOPLASMA GONDII*

By

W M HUTCHISON and K WORK

Received 24 iii 69

The two known forms of *Toxoplasma* are the trophozoites and the cystic forms the latter are sometimes referred to as zoites

The transmission of *Toxoplasma* cannot be solely due to infections by either the trophozoites or the cystic forms for reasons outlined by *Hutchison et al* (1969) It has become increasingly evident that another form or stage in the life history of *Toxoplasma* exists (*Hutchison et al* 1968) and the purpose of the present paper is to present in greater detail preliminary evidence in favour of this and to describe the events leading to the observation of a new encysted form by *Work & Hutchison* (1969)

MATERIALS AND METHODS

The two cats used in these experiments were purchased from different sources and were not related Both were females aged approximately six months They were helminth negative when obtained but were treated with an anthelmintic to remove any pre patent infections which may have been present Moreover at autopsy no *Toxocara* infections were observed in the alimentary tract The dye test titres of these cats prior to experimentation were unknown

The mice which were used in the preliminary experiments at Strathclyde were 3 months old and were offspring of a Strain A originally supplied to us 15 years ago by the Imperial Cancer Research Fund London We have examined the brains of several thousands of these mice which have not been subjected to any experimental procedures and have found none to be infected with the cysts of *Toxoplasma gondii* The strains of mice which are used at the Statens Seruminstitut are likewise *Toxoplasma* negative serologically and parasitologically

We are grateful to Professor *P C C Garnham* and Dr *J Chr Siim* for help and encouragement during this work We are also grateful to Dr *H Lautrop* in whose Department the bacteriological examinations of our inocula were made and to Dr *R S F Campbell* who carried out the histopathological examinations

Work at Strathclyde University is supported in part by grants from the *Medical Research Council Wellcome Trust* and *World Health Organisation* One of us (W M H) is indebted to *Carlsberg Breweries* and *The Statens Seruminstitut* for financial assistance which enabled him to work in Copenhagen Work at the Department of Toxoplasmosis and Viral Diseases Statens Seruminstitut is aided in part by grants to *J Chr Siim* from the *King Christian X Foundation* the *U S Public Health Service Research Grant Division (AI 01741)* Bethesda Md and the *World Health Organisation*

reduced during prolonged incubation with the labelled *E coli* The partly reversed inhibition was not observed when filtrates with more potent activities were used

It seems reasonable to assume that substances in the late filtrate might interfere with components of either antibody or complement character since both groups of serum components are believed to participate in the opsonization of *E coli* (7) as well as in the humoral destruction of the same bacteria (5)

It seems likely that the inhibitory effects of the late strangulation fluid on serum components may play a part in the pathophysiology of intestinal strangulation obstruction Interference with such components might favour local proliferation of bacteria in the peritoneal cavity and perhaps also spreading of bacteria to other organs One cannot however from these results conclude that the observed effects are responsible for the virulence enhancing property of the late strangulation fluid filtrate

SUMMARY

Phagocytosis of ³²P labelled *E coli* by rat PMN was determined *in vitro* Release of ³²P into the medium from the labelled bacteria was also determined Sterile filtrates of exudates from a strangulated loop of rat intestine appeared to inhibit the opsonic and ³²P releasing activity of rat serum when preincubated with serum at 37 C The inhibitory principles were present in filtrates of strangulation fluid collected during the late course of intestinal strangulation obstruction but were not detected in fluid collected during the early course of the ailment The findings suggest that the opsonic as well as the humoral bactericidal systems might be impaired during intestinal strangulation obstruction

REFERENCES

1 *Amundsen E* Studies on a toxicity enhancing factor in fluid from experimentally strangulated intestinal loops in the rat J surg Res *4* 531–536 1964
2 *Amundsen E & Midtvedt T* The toxicity of fluid from experimentally strangulated intestinal loops in the rat J surg Res *4* 306–313 1964
3 *Bornside G H Kuebler W J & Cohn I jr* Enhanced bacterial virulence in fluids produced in strangulation intestinal obstruction Proc Soc exp Biol (N Y) *121* 551–555 1966
4 *Mainland D* Elementary medical statistics 2 ed W B Saunders Company Philadelphia & London 1964 p 283
5 *Muschel L H* Serum bactericidal actions Ann N Y Acad Sci *88* 1265–1272 1960
6 *Roberts J & Quastel J H* Particle uptake by polymorphonuclear leucocytes and ascites carcinoma cells Biochem J *89* 150–156 1963
7 *Rowley D* Phagocytosis and immunity Experientia *22* 1 13 1966
8 *Spitznagel J K & Wilson L A* Normal serum cytotoxicity for P³² labeled smooth *Enterobacteriaceae* I Loss of label death and ultrastructural damage J Bact *91* 393–400 1966
9 *Trippestad A* Opsonizing and ³²P releasing activities of rat intestinal strangulation fluid on ³²P labelled *E coli* Acta path microbiol scand *77* 258–266 1969
10 *Trippestad A & Midtvedt T* Phagocytosis of ³²P labelled *E coli* by rat polymorphonuclear leucocytes Evaluation of a method *Acta path microbiol scand* *74* 259–273 1968

uniformly negative when the brains were examined for cysts 35 days later Separation 166 prepared from the faeces of the other cat during the first week after the initial toxoplasmic meal was also negative Separation 167 obtained during the second week from the same cat killed all of the six mice within eight days of the oral administration Microscopic diagnosis of toxoplasmosis from fresh brain smear at such an early stage is not easy but in three of these mice haemorrhagic lesions were observed and scattered about these lesions were small cyst like structures measuring 12–14 μ Closer examination of these cysts revealed well defined walls containing 2 6 crescentic organisms We were in no doubt that these were the newly formed cysts of *Toxoplasma gondii*

Histopathological examination did not reveal that other mouse pathogens were responsible for the deaths of these mice The passage mice into which the brains of the dead mice were sub cutaneously injected became *Toxoplasma* positive All controls for the experimental and passage mice were negative *Toxoplasma* infections were subsequently established in many other groups of mice using the same inoculum Separation 168 also produced toxoplasmosis in mice

TABLE 1

The Incidence of Infection with Toxoplasma gondii *in Groups of Mice Fed with Various Dilutions of a* Toxocara *negative Inoculum Prepared from Cat Faeces*

Inoculum dilution		No of mice fed with inoculum	No of mice with cysts in brain	Mortality of mice	Dye test on exptl mice Number positive/ survivors
Undiluted	a	6	6	4	2/2
	b	6	6	3	3/3
1/10	a	6	6	1	5/5
	b	6	6	0	6/6
1/20	a	6	6	1	5/5
	b	6	6	0	6/6
1/40	a	6	6	0	6/6
	b	6	6	0	6/6
1/80	a	6	6	0	6/6
	b	6	6	0	6/6
1/160	a	6	6	1	5/5
	b	6	6	0	6/6
1/320	a	6	6	1	5/5
	b	6	5	0	5/6

The cat had previously been fed with cystic toxoplasms in the tissues of infected mice for 6 days

a = antibiotics present in inoculum (20 000 units of penicillin + 10 mg of streptomycin/ml)

b = antibiotics absent.

Table 1 shows the results obtained by feeding the serially diluted inocula to groups of six mice Prior to feeding all these mice were sero negative having titres of less than 1 5 Eighty three of the eighty four mice used were *successfully infected* only one mouse (1 320 dilution) was found to be negative Seven of the eleven deaths among the experimental mice occurred with the undiluted inoculum Four of these seven mice were *in extremis* by the 10th day after feeding the inoculum and they were killed so that blood could be obtained for serological examination all were positive having a titre of 1 50 Thus early indications of the progress of the infection were obtained No lesions or cysts comparable to those observed in the Strathclyde mice were demonstrable on these occasions

The brains of all eleven mice which did not survive to autopsy were injected intra peritoneally into mice Serological and microscopical examination of these passage mice gave positive results indicating that the brains which had been injected had contained *Toxoplasma*

Thus in Table 1, Column 3 it will be realised that the information presented on the numbers of infected experimental mice is based on the microscopical detection of cysts in the brains of the mice which survived to autopsy and on brain passages in the cases of those mice which did not survive Serological data as indicated above were obtained on four of the latter which were killed because they were dying but for clearness of presentation this information and details of the passage results are excluded from Table 1 In Column 5 the numbers of sero positive experimental mice are expressed as ratios of the numbers surviving because this gives a more accurate impression of the sero positivity of each group

Apart from the undiluted inoculum the mortality of the mice in this experiment was not excessive Our fears of bacterial interference were not realised and it is obvious that the steps which were taken in an attempt to counteract this threat with antibiotics were unnecessary Blood agar cultures of the inoculum before and after ultra sonic disintegration indicated that this procedure greatly reduced the bacterial population The dispersed inoculum was cultured *anaerobically after the commencement of the experiment a growth of gram* positive motile as well as immotile rods resulted These were species of *Pseudomonas* None of the bacteria present were found to be pathogenic to mice

After passing the 1/10th dilution of the inoculum through a 35 micron stainless steel filter the filtrate could still produce *Toxoplasma* infections in mice thus giving some impression of the size of the infective unit

The results obtained from the experiment in which six mice were fed with a microscopically *examined Toxocara* negative Separation No 167 are given in Table 2 Only two of the six mice (Nos 5 and 6) survived to autopsy on the 48th day the dye test titres of both mice

had risen from less than 1 5 to 1 1250 Cysts were present in the brains of both mice Mouse No 1 3 and 4 died within 10 days of receiving the inoculum orally no serological examination could be carried out Mouse No 2 was dying and was killed on the 10th day its serum was positive having a dye test titre of 1 10 The brains of Mice Nos 1 4 were passaged into dye test negative groups which became positive Not all of the passage mice survived to autopsy but all which did survive were positive for *Toxoplasma*

TABLE 2
Dye Test Titres in Mice Fed with Faecal Separation No 167

Mouse number	Mouse survival in days after feeding	Dye test titre		Passage mice	
		Before feeding	At autopsy	Number positive	Number inoculated
1	8	<1 5	–	2	4
2	10	<1 5	1 10	4	4
3	9	<1 5	–	4	4
4	10	<1 5	–	3	4
5	48	<1 5	1 1250	–	–
6	48	<1 5	1 1250	–	–

All of the Separation fed to the mice had been microscopically examined and found to be negative for *Toxocara cati* ova

DISCUSSION

Hutchison (1965) *showed that faecal transmission could occur* After a cat was infected orally with the cysts of the Beverley Strain of *Toxoplasma* an aqueous inoculum was prepared from the cat faeces by flotation techniques This retained its toxoplasmic infectivity for a period of at least 12 months subsequent investigations extended this period of infectivity to 17 months after which time the inoculum became negative *In this inoculum* no living organisms were apparent apart from bacteria fungi coccidians and the ova of *Toxocara cati* No unknown protozoans were apparent in the inoculum and no form of *Toxoplasma* was at that time thought to be capable of surviving for such a long period outside of a host The evidence available at that time supported the idea that *Toxoplasma* and *Toxocara cati* were in *some way associated thus Hutchison* (1967) put forward the nematode transmission hypothesis It was suggested that the cystic forms liberated either within the stomach or intestine of the cat could be ingested by adult female *Toxocara cati* It was further suggested that the toxoplasms might be capable of penetrating the ova in these nematodes and thus pass to the external environment within them when *they were shed in the faeces of the cat Jacobs* (1967) and *Dubey* (1968) also supported this hypothesis The former showed that the sepa

rations after isolation from the cat faeces required a period of incubation before they became infective to mice It was thought that this non infective period might be correlated to the inability of the unembryonated nematode to hatch from the ovum The latter (*Dubey* 1967) removed larvae from their shells and micro isolated them Single larvae injected intra peritoneally into mice were incapable of causing toxoplasmosis but when two or more larvae were injected intra peritoneally several of the mice contracted *Toxoplasma* infections Thus the evidence up to that point seemed to fit the hypothesis

Jacobs (1967) was the first to report discrepant results with two cats *Toxocara* ova were not detected in the faecal inocula from these two animals despite the fact that both these could transmit *Toxoplasma* infections Both cats had been fed with *Toxocara* ova previously and he suggested that a light infection might have been missed at microscopical examination Against this he pointed out that no female *Toxocara* were evident at autopsy of the cats Jacobs felt that if the eggs were in fact absent we must resort to the hypothesis that a *developmental form of Toxoplasma* gondii is produced in the intestinal tract of cats fed the encysted protozoan

In Strathclyde we had noted that in some of our *Toxocara* incubations development of the ova had been arrested at the 8 cell stage Despite this one particular inoculum transmitted toxoplasmosis to mice This struck us as being peculiar since it is generally accepted that only a fully embryonated ovum is capable of hatching If the toxoplasm was also within the ovum we were puzzled as to how it could escape from the unembryonated nematode

We had carried out experiments on 32 cats at this stage Seventeen of the cats had been positive for *Toxocara* and after meals of chronically infected toxoplasmic mice fourteen of these passed faeces which were eventually proved to contain *Toxoplasma* positive material because they caused cyst formation when fed to mice We had been unsuccessful in transmitting infections with the fifteen remaining nematode negative cats

Two further *Toxocara* negative cats which are the subject of the present paper were investigated The experimental results described here indicate quite clearly that in at least one of the separations from the faeces of one of these cats forms of *Toxoplasma* were present because when this separation was fed to mice it produced rising *Toxoplasma* dye test titres and cysts in the brains We have also established that this *Toxoplasma* positive inoculum was *Toxocara* negative This was ensured not only by filtration experiments but also by microscopical examination of that part of the inoculum which was fed to the mice Because of the storage period of at least 1 month in tap water after the faecal separation the possibility that we might be recovering the conventional cystic forms from the faeces can be overruled Such cystic forms from the tissues can not survive for longer

than 30 minutes in water (*Jacobs et al* 1960) Moreover the properties of the faecal form which were outlined by *Hutchison et al* (1969) indicate that it is entirely different from the tissue form We concluded that there were unknown forms of *Toxoplasma* which could be passed in the faeces of cats and which were not associated with nematodes

We have subsequently been successful in repeating these experiments by isolating *Toxoplasma* from the faeces of a further five *Toxocara* negative cats in Glasgow and Copenhagen During the course of this work we (Work and Hutchison 1969) have isolated a resistant cyst which is capable of causing toxoplasmosis in mice However description and discussion of this new stage is outwith the scope of the present introductory paper but we are confident that our work will ultimately lead to more precise knowledge of the life history and taxonomic relationships of *Toxoplasma gondii*

SUMMARY

The isolation of *Toxoplasma gondii* from the faeces of a *Toxocara* negative cat which had been fed with toxoplasmic cysts in the tissues of infected mice indicates that a form of *Toxoplasma* exists which can be independent and unassociated with nematodes

ADDENDUM

Since the completion of this manuscript the work of *Dubey* (1968) has come to our notice and that of *Sheffield & Melton* (1969) and *Frenkel et al* (1969) has been published There seems to be no fundamental differences between our conclusions

REFERENCES

Angaard K Human toxoplasmosis The VIII International Congress of Paediatric Copenhagen 206–210 1960 (Ed J Chr Sum)

Dubey J P Studies on *Toxocara cati* larvae infected with *Toxoplasma gondii* J Protozool 14 Suppl p 4 1967

Dubey J P Feline toxoplasmosis and its nematode transmission Vet Bull Weybridge 38 495–499 1968

Dubey J P Isolation of *Toxoplasma gondii* from the faeces of a helminth free cat J Protozool 15 773–775 1968

Frenkel J K Dubey J P & Miller N L *Toxoplasma gondii* Fecal forms separated from eggs of the nematode *Toxocara cati* Science 164 432–433 1969

Hutchison W M Experimental transmission of *Toxoplasma gondii* Nature Lond 206 961 962 1965

Hutchison W M The nematode transmission of *Toxoplasma gondii* Trans R Soc trop Med Hyg 61 80–89 1967

Hutchison W M Dunachie J F & Work K The faecal transmission of *Toxoplasma gondii* Acta path et microbiol scandinav 74 462–464 1968

Hutchison W M Dunachie J F & Work K Transmissible Toxoplasms Symposium of the British Society for Parasitology VII 51–63 1969

Jacobs L *Toxoplasma* and toxoplasmosis Adv Parasitol 5 1–45 1967

Jacobs L Remington J S & Melton M L The resistance of the encysted form of *Toxoplasma gondii* J Parasit 46 11–21 1960
Sabin A B & Feldman H A Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoon parasite *(Toxoplasma)* Science *108* 660–663 1948
Sheffield H G & Melton M L *Toxoplasma gondii* Transmission through faeces in absence of *Toxocara cati* eggs Science *164* 431–432 1969
Work K & Hutchison W M A new cystic form of *Toxoplasma gondii* Acta path microbiol scandinav 75 191–192 1969

Acta path microbiol scand 77 283-290 1969

The Department of Microbiology and Immunology and the Department of Food Hygiene Veterinary College of Norway Oslo

INHIBITORY EFFECT OF LYSOLECITHIN ON BACTERIAL GROWTH

By

OLAV SANDVIK and TORE HØYEM

Received 18 ii 69

The role of lipids in the bacterial metabolism is in many ways less studied than other biochemical properties of the organisms However the decomposition of phospholipids is a well known ability of many bacteria (*Rossiter* 1967) As reviewed by *Klinge* (1957) *Esselmann & Liu* (1961) and *Bottcher* (1961) such abilities exist in a number of species in the genera *Clostridium*, *Bacillus* *Actinomyces* in several acid fast organisms and also in species of *Serratia* and *Vibrio* Phospholipase activity is also demonstrated among others in a coryneform organism (*Fossum & Hoyem* 1963) in *Staphylococcus aureus* (*Nygren et al* 1966 *Maheswaran & Lindorfer* 1967) and *Escherichia coli* (*Proulx & van Deenen* 1967)

In natural ecosystems like the animal body and various extraanimal environments microorganisms usually exist in the presence of more or less complex lipids It is probable that such ecosystems are influenced not only by the lipids originally present in the ecosystem but also by the lipid products that are formed and possibly accumulated during metabolism Thus it is observed (*Sandvik* unpublished) that certain strains of *Staphylococcus aureus* growing on egg yolk agar form metabolic products which gradually inhibit or destroy the organism itself This observation has stimulated the authors to examine whether particular products from lecithin catabolism may have a bactericidal effect The present paper deals with the bactericidal or bacteriostatic effect of lysolecithin on various organisms

MATERIALS AND METHODS

Strains

The organisms used are listed in Table 2 They were obtained from the American Type Culture Collection (ATCC) *Rockville* Maryland USA and from the culture collection of the Department of Microbiology and Immunology Veterinary College of Norway Oslo (NVH)

Requests for reprints should be addressed to *O Sandvik* Department of Microbiology and Immunology Veterinary College of Norway Oslo 4 Norway

Lysolecithin

The following products were used Lysolecithin (Sigma Grade II from egg lecithin) Lysolecithin (ex egg lecithin crystalline CHR) (Koch Light)§ Lysolecithin (NBC)†

All other chemicals used were of analytical grade When applied to bacterial media the lysolecithin wa as a rule dissolved in distilled water at 37 C

Media

Nutrient broth and nutrient agar (Difco) were used as basic media Blood agar was prepared by addition of defibrinated beef blood to a final concentration of 8 per cent (v/v) to melted and sufficiently cooled nutrient agar

Incubation

All cultures were incubated at 37 C

Preliminary Demonstration of the Growth Inhibiting Effect

In order to detect a possible bacteriostatic effect of lysolecithin or its catabolic products the growth ability of a number of organisms was tested on nutrient agar with lysolecithin applied to the surface An aqueous solutions of lysolecithin (5 mg/ml) was applied to particular areas of the agar surface in amount of approximately 0 03 ml per square centimeter After drying the treated agar surfaces the organisms to be tested were inoculated by streaking a loopful of young broth cultures (3-5 hours incubation) away from the untreated area of the agar surface The plates were read after 18–42 hours incubation by comparing the growth inside and outside the treated area

Semi Quantitative Determination of the Inhibiting Effect

Two different methods (I and II) were applied

By method I Petri dishes containing nutrient agar in 2 mm layers were used A segment of the agar layer was removed and replaced by pouring in a corresponding layer of nutrient agar containing the desired concentration of lysolecithin (Fig 1) The strains to be tested were inoculated from fresh blood agar cultures into tubes containing approximately 4 ml nutrient broth After 3 5 hours incubation streaks were made on the agar surface with a loopful of each culture The streaks were made perpendicular to the border between the agar layers from the untreated to the lysolecithin treated area of the agar After overnight incubation the plates were read as described for the preliminary examinations

By method II the basic media (nutrient agar or blood agar) were after melting and cooling to 45 C mixed with sufficient amounts of 3–4 hours broth cultures of the test organism to give an initial concentration of 10^5 cells/ml agar The agar was then poured on to rectangular framed glass plates giving 4 mm layers Wells were cut in the solidified agar by means of a 7 mm cork borer Aqueous serial dilutions of lysolecithin were made (Table 3) and 0 095 ml transferred from each tube to corresponding wells in the agar The plate were covered with tightly fitting glass lids and incubated overnight The inhibition titre of lysolecithin was determined as the highest dilution causing inhibition of the test organism around the well

When comparing the inhibitory effect in nutrient agar and blood agar the addition of blood was compensated by addition of a corresponding volume of nutrient broth to the non blood medium

Thin layer Chromatography

The purity of lysolecithin was checked by thin layer chromatography using commercial silica gel coated plates (Eastman Chromagram 6061) Chloroform methanol water (60 50 15) was used as solvent and the lysolecithin spot was stained by

Sigma Chemical Company St Louis Mo USA
§ Koch Light Laboratories Ltd Colnbrook Buckinghamshire England
† Nutritional Biochemical Corporation Cleveland Ohio USA
° Eastman Kodak Rochester N Y USA

TABLE 1

Growth of Different Organisms in the Presence of Lysolecithin on the Surface of Nutrient Agar

Organisms		Growth	Organisms		Growth
Staphylo occus aureus	(NVH 310)	—	*Staphylc oc us epidermid s*	(NVH 358)	—
Staphylo occus aureus	(NVH 465)	—	*Streptococcus agalactiae*	(NVH 2668)	—
Staphylococcus aureus	(NVH 2400)	—	*Streptococcus zooepi lemicus*	(NVH 2669)	—
Staphylococcus aureus	(ATCC 10832)	—	*Streptococcus faecalis*	(NVH 2671)	—
Staphylococcus aureus	(ATCC 11631)	—	*Listeria monoc ytogenes*	(NVH 2673)	+
Staphylococcus aureus	(ATCC 11632)	—	*Escherichia coli*	(NVH 2666)	+
Staphylococcus aureus	(NVH 199K)	—	*Salmonella typhimurium*	(NVH 2667)	+
Staphylo occus aureus	(NVH 4635K)	—	*Serratia marcescens*	(NVH 2672)	+
Staphyloc ccus aureus	(NVH 15 As)	—	*Pseu lomonas aeruginosa*	(NVH 2670)	+
Staphylo occus aureus	(NVH 5172K)	—	*Bacillus cereus*	(NVH 2665)	—
Staphylococcus aureus	(NVH 9470K)	—			
Staphylococcus aureus	(NVH 59 As)	—			
Staphylococcus aureus	(NVH 26(S)	—			
Staphylococcus aureus	(NVH 267S	—			
Staphylococcus aureus	(NVH 9(S)	—			
Staphylococcus aureus	(NVH 294S)	—			
Staphylococcus aureus	(NVH 95S)	—			
Staphylococcus aureus	(NVH 97S)	—			
Staphylococcus aureus	(NVH 259S)	—			
Staphylococcus aureus	(NVH 647S)	—			
Staphylococcus aureus	(NVH 589S)	—			
Staphylococcus aureus	(NVH 593S)	—			
Staphylcoc us aureus	(NVH 2661)	+			

+ Growth equal to that on untreated nutrient agar
— No growth.
Sigma NBC and Koch Light grades used with corresponding results Concentration of lysolecithin solution applied was 5 mg per ml distilled water

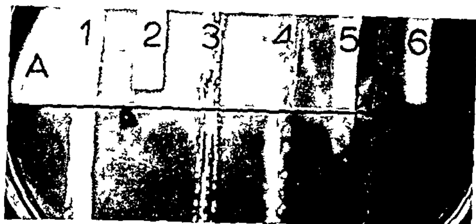


Fig 1

Growth inhibiting effect of lysolecithin on 5 different strains of *Staphylococcus aureus* (1 2 3 5 and 6) and 1 strain of *Bacillus cereus* (4) Streaks of the strains on the agar surface were directed from the plain nutrient agar (area A) to the lysolecithin containing (36 ppm) agar (area B) according to method I

spraying the chromatogram with a solution of 40 mg of bromothymol blue in 100 ml of 0 01 N sodium hydroxide (*Jatzkewitz & Mehl* 1960) or with 70 per cent perchloric acid—1M hydrochloric acid—4 per cent ammoniummolybdate (5 10 25) followed by exposure to ultraviolet light (*Hanes & Isherwood* 1949) Lysolecithin had an average R_f value of 0 35 in the chromatographic solvent employed

The chromatograms were performed in duplicate One plate was stained as described while the other after drying was transferred to a framed glass plate previously covered by a 2 mm layer of nutrient agar mixed with a 3 hours broth culture of *S aureus* (NVH 310) 7 parts nutrient agar to 1 part broth culture The thin layer plate was placed with the layer side against the agar surface care being taken to avoid air bubbles between the gel and the agar The plate was then covered by a lid and incubated overnight after which the thin layer plate was removed and the agar plate incubated for another 15 18 hours if necessary before examining the inhibition zones

RESULTS

Preliminary Demonstration of the Growth Inhibiting Effect

It may be seen from Table 1 that the growth ability for a considerable number of Gram positive organisms was distinctly inhibited by lysolecithin

Semi Quantitative Demonstration of the Inhibiting Effect

These examinations were usually performed according to method I (Fig 1) using a number of different strains of S aureus but also with single representatives of other species The results are listed in Table 2 The sensitivity of 3 strains of *S aureus* (NVH 310 NVH 465 NVH 2400) 1 strain of *Escherichia coli* (NVH 2666) and 1 strain of *Pseudomonas aeruginosa* (NVH 2670) to lysolecithin was tested after a one month interval representing at least 5 passages on blood agar with similar results

TABLE 2

Highest Concentration of Lysolecithin Allowing Growth of Different Organisms in Nutrient Agar (Method I)

Organisms		Concentration (ppm) of lysolecithin (Sigma) Full growth	Partly inhibited growth
Staphylococcus aureus	(NVH 310)	18	36
Staphylococcus aureus	(NVH 465)	72	
Staphylococcus aureus	(NVH 2400)	36	
Staphylococcus aureus	(NVH 59 As)	9	
Staphylococcus aureus	(ATCC 10832)	18	36
Staphylococcus aureus	(ATCC 11631)	9	
Staphylococcus aureus	(ATCC 11632)	18	36
Staphylococcus aureus	(NVH 2661)	>4570	
Streptococcus agalactiae	(NVH 2668)	4 5	9
Streptococcus zooepidemicus	(NVH 2669)	4 5	
Streptococcus faecalis	(NVH 2671)	18	36
Listeria monocytogenes	(NVH 2673)	1142	
Escherichia coli	(NVH 2666)	>4570	
Serratia marcescens	(NVH 2672)	>4570	
Pseudomonas aeruginosa	(NVH 2670)	>4570	
Bacillus cereus	(NVH 2665)	18	36–144

The following series was prepared 1 2 4 5 9 18 36 72 108 144 180 216 252 288 324 576 1142 2 84 4570 ppm

The Influence of Blood on the Growth Inhibiting Effect of Lysolecithin

The inhibiting effect of lysolecithin was tested by dripping solutions (144 ppm) of the phospholipid on to (a) nutrient agar and (b) nutrient agar with defibrinated beef blood added to a final concentration of 8 per cent (v/v) Two strains of *S aureus* (NVH 310 and NVH 465) and one strain of *Streptococcus faecalis* (NVH 2671) were used as test organisms All conditions regarding application of the phospholipid inoculations of test strains and reading of plates were as described for the preliminary tests These experiments showed that the growth inhibiting ability of lysolecithin which was total on nutrient agar plates seemed to be completely neutralized on blood agar plates Corresponding results were obtained when the same strains were tested against lysolecithin (final concentration 72 ppm) according to method I in the absence and presence of blood

With *S aureus* (NVH 310) as test organism semi quantitative titrations of the anti bactericidal effect of blood were performed according to method II It can be seen from Table 3 that the growth inhibiting effect of lysolecithin was about 16 times higher on plain nutrient agar than on blood agar

Other protein containing fluids such as haemolyzed erythrocytes or blood serum also showed an anti bactericidal effect against lysolecithin but quantitative analyses were not carried out in these cases Heat

treatment of lysed erythrocytes at (a) 60 C for 2 hours and (b) 65 C for 20 minutes did not significantly alter the anti bactericidal effect More extreme heat treatment was not carried out due to coagulation of the protein

TABLE 3

Lowest Concentration (ppm) of Lysolecithin Causing Inhibition Zones against Staphylococcus aureus (NVH 310) in Nutrient Agar and Blood Agar (Method II)

	Nutrient agar	Blood agar
Lowest effective concentration	72	1152

The following dilution series of lysolecithin (Sigma) was prepared 18 36 72 144 288 576 1152 and 1274 ppm.

Thin Layer Chromatography

The lysolecithin preparation used in this study appeared to consist of one single compound as judged from the thin layer chromatography experiments Only one spot could be detected using any of the staining methods described The technique of using microbiological location of the lysolecithin spot is an independent method indicating distinctly the presence of growth inhibiting substances on the thin layer plate Only one such substance could be detected possessing the same R_f value as lysolecithin

DISCUSSION

The examinations indicate that lysolecithin possesses a considerable growth inhibiting effect against certain bacteria The limit of tolerance varied from 4 5 ppm for the most sensitive streptococci to over 4600 ppm for all the Gram negative organisms tested The variation in strain tolerance from 9 ppm to over 1500 ppm between the most sensitive and the most resistant strains of *S aureus* is very striking The strain of *Listeria monocytogenes* included was surprisingly resistant (tolerance greater than 1100 ppm) as compared with other Gram positive organisms of similar growth ability for example streptococci The high resistance of this organism to lysolecithin is interesting in relation to its preference for lipid containing tissues like the brain

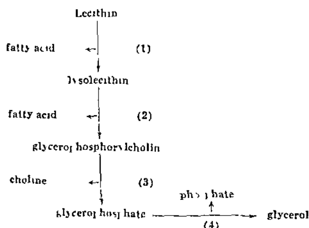
It is known that lysolecithin may be highly toxic to animal cells Thus this phospholipid acts strongly lytically on erythrocytes (*Habermann & Mølbert* 1954) and it is also observed to disrupt the mitochondria in liver cells (*Nygaard et al* 1954) An immobilizing and inactivating effect of lysolecithin on spermatozoon is also described (*Iritani & Nishikawa* 1962 and 1963 *Aamdal et al* 1965) However the exact mechanism of the toxicity of lysolecithin seems to be uncertain but part of the effect may be due to the high surface activity of the phospholipid (*Neumann & Habermann* 1957)

The strong bacteriostatic effect of lysolecithin gave rise to the ques

tion of whether the commercial chemicals used could be contaminated with for instance antibiotics It was however clearly demonstrated by thin layer chromatography that the growth inhibiting component of lysolecithin exhibited mobility values identical with that of the lyso lecithin itself It should be mentioned that corresponding inhibiting effects occurred when different organisms were tested with lysolecithin obtained from different manufacturers (Table 2) The chromatography experiments indicate that the lysolecithin preparations employed were pure and did not contain other inhibitory substances

It is interesting that one or more blood factors may neutralize the growth inhibiting effect of lysolecithin Whether this ability is due to a direct detoxifying activity of the blood components or to an enzymatic decomposition of the phospholipid remains an unsolved problem The antagonistic effect of blood components is important in that they may bring about a partial neutralization of the lysolecithin activity in the animal organism Thus the antagonism mav be of significance with regard to the infectivity of certain microorganisms in animal ecosystems

A decomposition of lecithin via lysolecithin may be due to microbial activity as follows



Reaction 1 is catalized by phospholipase A As demonstrated by *Nygren et al* (1966) certain strains of *S aureus* may produce a phospholipase A Accordingly these organisms may in the presence of lecithin bring about a production of lysolecithin If lysolecithin decomposing enzymes or detoxifying compounds are absent from the environment a harmful accumulation of lysolecithin may occur It is not clear if this type of accumulation may explain the observed self destroying activity occurring when certain strains of staphylococci are grown on egg yolk agar (*Sandvik* unpublished)

In conclusion the presence of phospholipids seems to be able to

cause complicated conditions both in animal and in extraanimal bacterial ecosystems

SUMMARY

A growth inhibiting effect of lysolecithin has been demonstrated for a number of different bacteria The most sensitive organisms examined for example streptococci were not able to grow in the presence of 4–5 ppm lysolecithin in agar media while the most resistant organisms like enterobacteria grew well at concentrations greater than 4500 ppm Strain differences in sensitivity to the phospholipid were examined for some *Staphylococcus aureus* strains The extreme tolerance limits were 9 ppm and greater than 4500 ppm for the most sensitive and the most resistant strain respectively The tested strain of *Listeria monocytogenes* was resistant to more than 1100 ppm lysolecithin and thus surprisingly tolerant as compared with other Gram positive organisms of similar growth ability like streptococci An unidentified blood component was shown to neutralize the growth inhibiting effect of lysolecithin This compound was found to be relatively heat stable The significance of the growth inhibiting effect of phospholipids in different ecosystems is discussed

REFERENCES

Aamdal J Lyngset O & Fossum K Toxic effect of lysolecithin on sperm Nord Vet Med *17* 633–634 1965

Bottcher K Lecithinase activity of some sporogenous soil bacteria Mikrobiologiya *30* 673–678 1961

Esselmann M T & Liu P V Lecithinase production by Gram negative bacteria J Bact *81* 939–945 1961

Fossum K & Høyem T Phospholipase D activity in a non haemolytic coryneform bacterium Acta path microbiol scand *57* 295–300 1963

Habermann E & Molbert F Zur morphologischen Differenzierung der Hamolyse durch Bienengift Schlangengift Lysocithin und Digitonin Naunyn Schmiedeberg s Arch exp Path Pharmak *223* 203–216 1954

Hanes C S & Isherwood F A Separation of the phosphoric esters on the filter paper chromatogram Nature *164* 1107–1112 1949

Iritani A & Nishikawa Y Studies on the egg yolk coagulating factor (enzyme) in goat semen III Coagulating action to the fractionated yolk Zootecn e Vet *17* 392–395 1962

Iritani A & Nishikawa Y Studies on the egg yolk coagulating factor in goat semen III IV V Jap J anim Reprod *8* 109–121 1963

Jatzkewitz H & Mehl E Thin layer chromatography of brain lipides hydrolytic and breakdown products Hoppe Seylers Z physiol Chem *320* 251–257 1960

Klinge K Differenzierung der im Wasser vorkommenden Bakterien des Genus Pseudomonas durch die Eigelbreaktion Arch Hyg (Berl) *141* 348–360 1957

Maheswaran S K & Lindorfer R K Staphylococcal β hemolysin II Phospholipase C activity of purified β hemolysin J Bact *94* 1313–1319 1967

Neumann W & Habermann E Clearing action of lysolecithin Nature *180* 1284 1957

Nygaard A P Dianzani M U & Bahr G F The effect of lecithinase A on the morphology of isolated mitochondria Exp Cell Res *6* 453–458 1954

Nygren B Hoborn J & Wahlen P Phospholipase A production in Staphylococcus aureus Acta path microbiol scand *68* 429–433 1966

Proulx P R & van Deenen L L M Phospholipase activities of Escherichia coli Biochim biophys Acta (Amst) *144* 171–174 1967

Rossiter R J Biochemistry of phosphatides In Schettler G (Ed) Lipids and lipidoses Springer Verlag Berlin Heidelberg New York 1967 p 93–121

Sandvik O Unpublished work

Acta path microbiol scand 77 291–298 1969

Institute of Medical Microbiology University of Lund Lund Sweden

AN ACTIVATOR OF C'1s TO C'1 ESTERASE IN THE MACROGLOBULIN FRACTION OF HUMAN SERA

By

ANNA BRITA LAURELL

Received 31 iii 69

In a study concerning the activation of C'1 to C'1a of euglobulin preparations two types of activation was shown (*Lepow et al* 1965) The one type of activation was not influenced by C'1 esterase inhibitor and was concidered as a spontaneous slow process The other reaction was an autocatalytic activation of C'1 which was inhibited by C'1 esterase inhibitor

Hereditary angioneurotic edema (HANE) is accompanied by a hereditary deficiency of C'1 esterase inhibitor (*Donaldson & Evans* 1963) But this deficiency cannot explain the activation of C'1 to C'1 esterase in these patiens as the C'1 esterase inhibitor does not interfere in the primary activation process

In the present investigation HANE sera sampled in attack free periods and containing small amounts of C'1 esterase inhibitor and normal sera respectively were fractionated by gel filtration on Sephadex G 200 in the presence of EDTA Individual fractions and combination of fractions were analysed for C'1 esterase activity by measuring the C'4 destroying activity Evidence of a factor activating C'1 and C'1s to C'1 esterase was obtained In this paper the term C'1 esterase is used to designate both C'1a and activated C'1s

MATERIALS AND METHODS

Blood was sampled from healthy individuals and f om patients with HANE. The samples were centrifuged within 2 hours and the serum frozen immediately at −80 C in aliquots of 4 ml The HANE sera were prepared from blood samples obtained from 4 patient during periods free from attacks The HANE sera contained 2 5–3 5 Units C'1 *esterase inhibitor* per ml No C'1 esterase as measured by ATEe (N acetyl L tyrosineethyl ester) hydrolyzing capacity (*Levy & Lepow* 1959 *Laurell et al* 1965) was demonstrable until after the sera had been heated at 37 C for 90 to 100 minutes

---

This investigation was supported by grants from the *Swedish Medical Research Council* (project No B 69 16 X 63 05 A)

Address Institute of Medical Microbiology Solvegatan 23 University of Lund Lund Sweden

C'1 esterase inhibitor was prepared according to *Pensky et al* (1961)

Gel filtration on Sephadex G 200 was performed as described by *Laurell & Siboo* (1966)

C'4 destroying activity was determined according to *Laurell & Siboo* (1966) Serum heated at 56 for 20 minutes was used a source of C 4 The amount of C'1 esterase inhibitor in the dose used in the test was ≷ 0 2 U/ml

Electrophoresis in antibody containing agarose gel according to Laurell C B (1966) with the use of a specific rabbit anti human C 1 esterase inhibitor *erum* The procedure used for preparation of the antiserum and estimation of the amount of C 1 esterase inhibitor will be published elsewhere (*Laurell et al* to be published)

RESULTS

Sera from four patients with HANE were fractionated by gel filtration on Sephadex G 200 *in the presence of LDTA 10 ³M No C 4 destroying* activity was found with 0 1 ml of the fractions—the largest dose tested—after incubation for 1 or 2 hours C 4 destroying activity *appeared when fractions from peak I and fractions located between* peaks II and III in amounts of 0 0125 to 0 05 ml were pooled and incubated at 37 C for 1 or 2 hours (Fig 1 and Table 1)

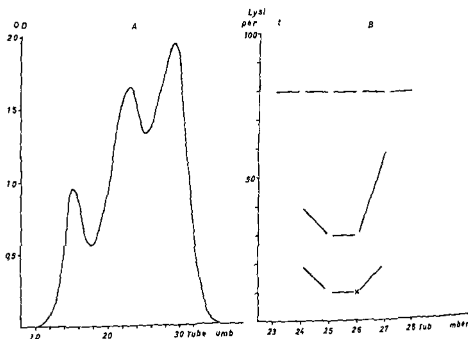


Fig 1

C 4 destroying activity of combined fractions obtained after gel filtration on Sephadex G 200 of HANE serum R I 1 Protein pattern *obtained after gel filtration* on Sephadex G 200 *B The recalcified fractions located between peak II and III (ab* scissa) were combined with fractions 16 *of peak I in volumes of* 0 0⁰5 and 0 0125 ml, respectively and incubated for 2 hours at 37 C The C 4 destroying activity of the combinations were determined No C 4 *destroying activity* was demonstrated in any of the fractions in volumes of 0 1 ml when tested alone ×——× fraction 16 0 0⁰5 ml — — fraction 16 0 01⁰5 ml O——O veronal buffer 0 1 ml

TABLE 1

C 4 Destroying Activity of Combined Fractions Obtained after Gel Filtration on Sephadex G 200 of HANE Serum I H

	0 05 ml of fractions number						0 05 ml Veronal buffer
	23	24	25	26	27	28	
0 05 ml fraction 15	90	80	60	20	20	20	100
16	40	10	0	0	0	20	100
17	10	10	0	0	0	20	100
18	20	10	0	0	0	20	100
0 05 Veronal buffer	100	100	100	100	100	100	100

Incubation of the combined fractions with C 4 for 20 minutes at 37 C R4 and ensitized sheep cells were added and the percentage of hemolysis was recorded after incubation of the mixture at 37 C for 30 minutes

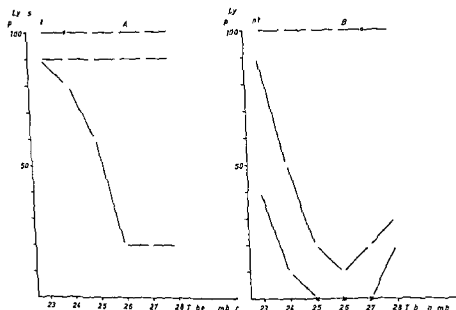


Fig 2

C 4 destroying activity of combined fractions obtained after gel filtration on Sephadex G 200 of HANE serum I H The fractions located between peak II and III (abscissa) were combined with fractions 15 (A) and fraction 16 (B) recalcified and not recalcified No C 4 destroying activity was demonstrable in any single fraction when tested alone X——X combined fractions (0 05 ml of each) recalcified — — combined fractions (0 05 ml of each) not recalcified O——O veronal buffer 0 1 ml

Recalcified and non recalcified individual fractions and combinations of them were incubated at 37 C and tested Recalcification resulted in a higher C 4 destroying activity in combinations of fractions from peak I and fractions located between peaks II and III (Fig 2) No

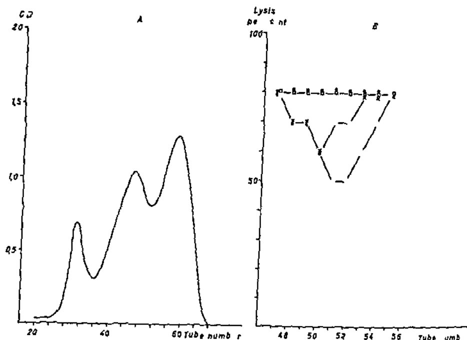


Fig 3

C 4 destroying activity of combined fractions obtained after gel filtration on Sephadex G 200 of normal human serum *A* Protein pattern obtained after gel filtration on Sephadex G 200 *B* The fractions located between peak II and III (abscissa) were combined with fractions 30 33 and 36 of peak I respectively No C 4 destroying activity was demonstrated in any single fraction when tested alone X——X fraction 30 0 05 ml — — fraction 33 0 05 ml O——O fraction 36 0 05 ml □——□ veronal buffer 0 1 ml

single fraction incubated separately at 37 C before the test for the same time as the fractions combined contained demonstrable C 4 destroying activity—not even in eight fold volumes

When the fractions obtained after gel filtration of normal sera were combined with the fractions obtained from HANE sera C 4 destroying activity appeared in the same combinations as with the reciprocal HANE fractions C 4 destroying capacity was demonstrated in combination of HANE peak I fractions with the fractions of normal sera located between peak II and III However this combination was less effective than that of HANE peak I with HANE fractions of the peak *II—III* The reversed combination first peak fractions from normal serum and HANE fractions from the area between peak II—III resulted in a weak C 4 destroying activity

When the corresponding fractions from normal sera were combined and incubated for 4 hours at 37 C a low but well demonstrable C 4 destroying activity appeared (Fig 3) In this experiment a *higher column* (90 cm) was used for the filtration

The comparatively small amount of C 1 esterase which generated on

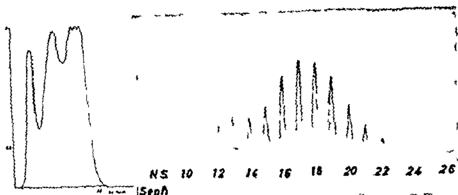


Fig 4

Localization of C1 esterase inhibitor by electrophoresis in antibody containing agarose gel (to the right) of fractions obtained after gel filtration of normal human serum on Sephadex G 200 (Left protein pattern)

combination of fractions of peak I from HANE sera with fractions of normal sera located between peak II and III might be explained by the presence of C1 esterase inhibitor in the latter fractions of normal sera The fractions of normal serum obtained after gel filtration were therefore investigated for the presence of C1 esterase inhibitor with electrophoresis in antibody containing agarose gel using a specific anti C1 esterase inhibitor antiserum (Fig 4)

The C1 esterase inhibitor was located in peak II and trailed in the area where C1s was eluted However C1 esterase inhibitor was situated also in peak I

DISCUSSION

C1 in serum is a 19 S molecule which in the presence of EDTA splits into three subunits C1q C1r and C1s These subunits can be separated in three distinct fractions by chromatography on DEAE cellulose C1s is a proenzyme which is transferred to an esterase when C1 is activated to C1a (macromolecular C1 esterase) (*Lepow et al* 1963)

On gel filtration on Sephadex G 200 of normal human serum C1 esterase activity appears in the macroglobulin peak (*Laurell & Siboo* 1966) When gel filtration is performed in the presence of EDTA no C1 esterase activity could be shown in any of the fractions C1 esterase was found in the macroglobulin peak of HANE sera After gel filtration of such sera in the presence of EDTA C1 esterase was recovered not in the macromolecular fractions but in fractions located between peak II and III The results indicate that C1s is located in the fractions between peak II and III (*Laurell & Siboo* 1966)

The present investigation aimed at revealing a possible activator of C1 in serum by fractionation of HANE sera and normal sera by gel filtration on Sephadex G 200 in the presence of EDTA The HANE sera

obtained in attack free periods contained small amounts of C1 esterase inhibitor and showed no C1 esterase activity C1 esterase activity measured as C4 destroying activity generated on combination of fractions belonging to peak I and fractions located between peak II and III None of these fractions were active alone not even in volumes 8 times as large as those found to be active when combined These results indicate the presence in the macromolecular fractions of an activator of C1s to C1 esterase

The activator proved more effective in presence of calcium ions This seems to be compatible with the findings of a slower activation of C1 to C1 esterase in serum and plasma from a patient with HANE when the EDTA concentration of the test solution was increased (*Lundh et al* 1968)

Evidence was also produced of the occurrence of a C1 activator in the macroglobulin fraction of normal human serum The C1 esterase activity that generated when fractions from the macroglobulin peak of normal sera were combined with C1s containing fractions of normal sera was lower than that developed when the latter fractions were pooled with the macroglobulin fraction from HANE sera Whether this means that the concentration of the factor of normal serum is lower than that in HANE sera or that the two types of sera contain different activators cannot be decided from the present experiments

The difference of C1 esterase activity generated on combination of peak I fractions of HANE sera with fractions located between peak II and III of normal sera and of HANE sera respectively might well be explained by the presence of small amounts of C1 esterase inhibitor in the fractions of normal sera containing C1s Activation of C1s then probably occurs but C1 esterase is partially masked by the presence of C1 esterase inhibitor

Ratnoff et al (1967) showed that highly purified C1s was activated to C1 esterase by trypsin and plasmin On addition to normal human serum of trypsin in amounts not exceeding the inhibiting effect of the trypsin inhibitors in serum C1 esterase is generated and C1 esterase inhibitor is consumed (*Laurell* 1968) It has been shown that C1 in HANE plasma can be activated by the Hageman factor (*Donaldson* 1968a) and that urokinase hastens the activation of C1 esterase in serum and plasma from persons with HANE (*Donaldson 1968b*) *Gigli et al* (1968) have showed an increase of the esterolytic and hemolytic activity of C1 after exposure to kallikrein This observation is of interest first because the C1 esterase inhibitor inhibits also kallikrein (*Kagan & Becker* 1963) and second because *Landerman et al* (1962) have shown kallikrein to be the factor eliciting the symptoms in patients with HANE It would thus appear that the complement system and other enzymes of serum and plasma *e g* kallikrein can influence each other Enzymes released or activated for one or other reason (trauma cell damage stress) may result in an activation

of C1 to C1 esterase which may in turn act on the subsequent complement factors

The possibility existed that small amounts of C1 in macromolecular form or C1s are in some way activated during gel filtration and that a combination of fractions results in an autocatalytic activation of C1 esterase But the findings in the present study argued against such a possibility because when tested separately the fractions used in the experiments with combinations had no demonstrable C1 esterase activity not even when used in eight fold volumes The less effective generation of C1 esterase on combination of fractions in the absence of calcium ions also argues against an exclusively autocatalytic process Divalent cations do not influence the enzymatic function of C1 esterase (*Haines & Lepow* 1964) It therefore seemed reasonable to assume that the factor shown in HANE sera and also in normal sera was not C1 esterase

In preliminary studies on purified C1 esterase inhibitor the inhibitor was eluted in the peak II fractions after gel filtration on Sephadex G 200 (*Laurell* to be published) The finding of C1 esterase inhibitor not only in the second peak but also in the macromolecular fractions of normal sera after gel filtration (Fig 4) might indicate a complexing of the inhibitor with other serum constituents *e g* kallikrein C1r or activated C1s This finding is receiving further attention

SUMMARY

1 On gel filtration on Sephadex G 200 in the presence of EDTA of HANE sera containing no active C1 esterase and of normal human sera no C1 esterase activity was demonstrated in the separated fractions

2 When the fractions of the macroglobulin peak and the fractions eluted between the second and third protein peak were pooled and incubated at 37 C C1 esterase activity appeared Such activity appeared on combination of the fractions of HANE sera of fraction of normal sera as well as on combination of fractions of both two types of sera

3 More C1 esterase was formed in the combinations in the presence of calcium ions

4 The findings indicated the presence in the macroglobulin fraction obtained after gel filtration in the presence of EDTA of an activator of C1 and C1s to C1 esterase

REFERENCES

1 [illegible] Mechanisms of activation of C1 esterase in hereditary angioneurotic edema plasma in vitro J exp Med [illegible] 1968a

2 [illegible] C1 activation in hereditary angioneurotic edema plasma role of [illegible] J Appl Physiol [illegible] 1968b

Donaldson V H & Evans R R A biochemical abnormality in hereditary angioneurotic edema Absence of serum inhibitor of C1 esterase Amer J Med 35 37–44 1963
Gigli I Mason J W Colman R W & Austen K F Interaction of kallikrein with the C1 esterase inhibitor J Immunol *101* 814–815 1968
Haines A L & Lepow I H Studies on human C1 esterase I Purification and enzymatic properties J Immunol 92 456–467 1964
Kagen L J & Becker E L Inhibition of permeability globulins by C1 esterase inhibitor Fed Proc 22 613 1963
Landerman N S Webster M F Becker E I & Radcliffe H E Hereditary angioneurotic edema II Deficiency of inhibitor for serum globulin permeability factor and/or plasma kallikrein J Allergy 33 330–341 1962
Laurell A B Effect of trypsin on complement components in normal human serum. Acta path microbiol scand 72 139–146 1968
Laurell A B Lundh B & Malmquist J Inability of highly purified streptokinase preparation to inactive complement in serum Acta path microbiol scand 64 318 328 1965
Laurell A B & Siboo P Activation of C1 to C1 esterase on gel filtration on Sephadex G 200 Acta path microbiol scand 68 230–242 1966
Laurell C B Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies Analyt Biochem 15 45–52 1966
Lepow I H Naff G B Todd E W Pensky J & Hinz Jr C F Chromatographic resolution of the first component of complement into three activities J exp Med *117* 983–1008 1963
Lepow I H Naff G B & Pensky J Mechanism of activation of C1 and inhibition of C1 esterase CIBA Foundation Symposium Complement p 74–90 Churchill London 1965
Levy L R & Lepow I H Assay and properties of serum inhibitor of C1 esterase Proc Soc exp Biol (NY) *101* 608 611 1959
Lundh B Laurell A B Wetterqvist H White T & Granerus G A case of hereditary angioneurotic oedema successfully treated with ε aminocaproic acid Studies on C1 esterase inhibitor C1 activation plasminogen level and histamin metabolism Clin exp Immunol 3 733–745 1968
Pensky J Levy R & Lepow I H Partial purification of a serum inhibitor of C1 esterase J Biol Chem 236 1674–1679 1961
Ratnoff O D & Naff C B The conversion of C1s to C1 esterase by plasmin and trypsin J exp Med *125* 337–358 1967

Acta path microbiol scand 77 299-308 1969

WHO International Escherichia Centre Statens Seruminstitut
Copenhagen Denmark

STUDIES ON THE ANTIGENIC STRUCTURE OF SEX FIMBRIAE* CARRIED BY A STRAIN OF *SHIGELLA FLEXNERI* 4b

By

I KÉTYI° and I ØRSKOV

Received 24 iv 69

An antigen termed f characteristic for F and Hfr *E coli* strains was demonstrated in 1960 (26) Later strong correlative evidence was obtained for the identity of the f antigen and the F fimbria (11 8) It was also shown that the R factor R100-1 a depressed mutant of the fi factor R100 controlled the synthesis of an antigenic substance serologically related to the f antigen (7) However the extent of this relationship was difficult to elucidate as auto agglutinability always accompanied the R100 1 factor in the *E coli* strain examined and thus disqualified the strain for bacterial agglutination tests The fact that bacteria which harbour an R factor of the fi+ class are sensitive to F specific phages is consistent with the antigenic relationship (17 18) and in the electron microscope the presence of fimbriae resembling F fimbrae has been shown (3) However some R factors of the F like class (at least R100 1) determine specific fimbriae which differ to some extent from F fimbriae in their affinity for F specific RNA phages (21)

The I fimbria is another example of af fimbria determined by a transmissible plasmid the col I factor It is morphologically and antigenically different from the F fimbriae and absorbs I but not F specific phages (15) The K88 antigen is a further example of a fimbrial antigen the determinant of which is transmissible (20 23 27)

Lawn Meynell Meynell & Datta (13) have suggested that such fimbriae should be called sex fimbriae as opposed to various types of common fimbriae found on many Enterobacteriaceae

In a previous report (9) the identity of the f antigens carried by *E coli* and *Sh flexneri* F strains was shown The present paper provi

* The term fimbriae originally proposed by *Duguid* (4) is employed in this study instead of pili (2)

° Present address Institute of Microbiology University Medical School Pécs Hungary

The authors wish to express their sincere thanks to *G G Meynell* (London) and *C Hausmann* (Dallas Texas) for kind provision of bacterial and phage strains

Donaldson V H & Evans R R A biochemical abnormality in hereditary angioneurotic edema Absence of serum inhibitor of C1 esterase Amer J Med 35 37–44 1963
Gigli I Mason J W Colman R W & Austen K F Interaction of kallikrein with the C1 esterase inhibitor J Immunol *101* 814–815 1968
Haines A L & Lepow I H Studies on human C1 esterase I Purification and enzymatic properties J Immunol *92* 456–467 1964
Kagen L J & Becker E L Inhibition of permeability globulins by C1 esterase inhibitor Fed Proc 22 613 1963
Landerman N S Webster M E Becker E L & Radcliffe H F Hereditary angioneurotic edema II Deficiency of inhibitor for serum globulin permeability factor and/or plasma kallikrein J Allergy *33* 330–341 1962
Laurell A B Effect of trypsin on complement components in normal human serum Acta path microbiol scand 72 139–146 1968
Laurell A B Lundh B & Malmquist J Inability of highly purified streptokinase preparation to inactive complement in serum Acta path microbiol scand 64 318–328 1965
Laurell A B & Siboo R Activation of C1 to C1 esterase on gel filtration on Sephadex G 200 Acta path microbiol scand 68 230–242 1966
Laurell C B Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies Analyt Biochem *15* 45–52 1966
Lepow I H Naff G B Todd E W Pensky J & Hinz Jr C F Chromatographic resolution of the first component of complement into three activities J exp Med *117* 983–1008 1963
Lepow I H Naff G B & Pensky J Mechanism of activation of C1 and inhibition of C1 esterase CIBA Foundation Symposium Complement p 74–90 Churchill London 1965
Levy I R & Lepow I H Assay and properties of serum inhibitor of C1 esterase Proc Soc exp Biol (N Y) *101* 608–611 1959
Lundh B Laurell A B Wetterqvist H White T & Cranerus G A case of hereditary angioneurotic oedema successfully treated with ε aminocaproic acid Studies on C1 esterase inhibitor C1 activation plasminogen level and histamin metabolism Clin exp Immunol 3 733–745 1968
Pensky J Levy R & Lepow I H Partial purification of a serum inhibitor of C1 esterase J Biol Chem 236 1674–1679 1961
Ratnoff O D & Naff G B The conversion of C1s to C1 esterase by plasmin and trypsin J exp Med *125* 337–358 1967

The antisera against strains with plasmids were prepared according to the method described previously (26) for the production of F antisera, except that formalin treated culture was used as antigen instead of live culture

Antigen antibody reactions Slide agglutination tests were made with 1 10 or 1 20 antiserum dilutions and broth agar cultures incubated overnight at 37 C Tube agglutination was performed with two fold serial dilutions of antisera and freshly prepared saline suspensions of overnight broth agar cultures Readings were made after 24 hours incubation at 37 C

It should be noted that the fimbrial type of agglutination is always very finely granulated and is particularly difficult to read in the case of the R 100 1 or colIb carrying strains

Absorption of antisera was made by the method described by *Ørskov* (26)

Phage sensitivity tests These experiments were performed with phages f1 and ff1 by the overlayer method (1) using broth cultures incubated in a rotator for 2 hours at 37 C The medium described by *Loeb & Zinder* (15) was used for phage dilutions

Transfer of plasmids and selection procedures The F transfer method used was described in a previous paper (9) The isolation of the *Shigella* recipient was guided by differences in fermentation between donor and recipient on EMB sugar plates At least 100 colonies were isolated

The presence of the episomes F F and K88 was tested by slide agglutination The successful transfer of R factor was demonstrated by the acquired resistance to antibiotics and the col factors B2 and I1a on the basis of production of colicin

Antibiotic resistance tests The resistance to antibiotics and the selection of R *Shigella* derivatives were demonstrated on broth agar plates containing 100 μg/ml streptomycin chloramphenicol or tetracycline using the replica plating technique (14)

Quantitative tests were made in broth by means of the dilution technique

Colicin production The method described by *Frédéricq* (5) was used for detection of colicin producing colonies

Media EMB plates containing 1 per cent sugar were used except in the case of maltose where the sugar content was 1 5 per cent

Ox heart broth infusion was used as fluid medium and solidified with 1 6 per cent agar for ordinary use and 0 6 per cent in soft agar layer In all cases 1 per cent peptone 0 3 per cent NaCl and 0 2 per cent Na_2HPO_4 $12H_2O$ were added

The minimal agar supplemented by amino acids was described in a previous paper (9)

RESULTS

Transfer of the Various Plasmids to Sh Flexneri 4b

The F and F13 lac factors Transfer of the F factor was described previously (9) In those experiments 1–5 per cent of reisolated *Sh flexneri* 4b colonies agglutinated in an *F coli* F antiserum

In the experiments reported here F factor F13 lac was transferred from the K12 strain W 3287 to the same *Sh flexneri* 4b recipient strain also by overnight mixed cultivation Recipient colonies that had received the lac character were reisolated on suitably supplemented minimal lactose plates (for contraselection of the donor) All such colonies agglutinated in an F antiserum and segregated lac colonies with a frequency of about 10 ³ thus pointing to the fact that the colonies had arisen as a result of diploid zygosis i e that no lac recombination had taken place The percentage of recipient colonies that received the F13 lac factor was only 0 001 which is a much lower percentage than in the case of F transfer However these figures might not be comparable as the F colonies were selected on a complete medium and the F13 lac colonies on a minimal medium with lactose as carbon source

The R100 1 factor This factor a depressed mutant of R100 which confers resistance to streptomycin chloramphenicol tetracycline and sulphathiazole was transferred from the K12 strain D368 (also numbered Je 170 (7)) The strain carries in addition a chromosome located streptomycin resistance After overnight mixed cultivation the *Sh flexneri* 4b strain was re isolated on EMB mal plates containing 100 µg streptomycin/ml About 2 per cent of the recipient cells grew on this medium One purifed recipient clone D4301 was examined further As the donor *E coli* strain it was resistant against 1000 µg streptomycin 500 µg chloramphenicol and 250 µg tetracycline per ml A high level of resistance to sulphathiazole had already been demonstrated in the *Shigella* strain before introduction of the R factor The streptomycin resistance carried by the *R100 1* factor normally gives a lower level of resistance in an *E coli* strain than in a *Shigella* culture In this case however this lower level of resistance to streptomycin was masked by the chromosome determined high level resistance in the *E coli* strain

The colB2 factor The derepressed *(fdr) mutant of the colB2 factor* carried by the *E coli* K12 strain CH99 (6) was transferred into a colB2 resistant mutant D4470 of the *Sh flexneri* 4b strain which served as recipient in all transfer experiments Re isolation after mixed cultivation was carried out on EMB xyl plates Examination of colicin production on broth agar plates showed that 44 per cent of recipient colonies had acquired this ability

The colE1a factor This derepressed factor known to be associated with an I like sex factor *(19)* was transferred from the *S typhimurium* strain M533 to Sh flexneri 4b using a similar method to that mentioned above 10 per cent recipient colonies produced colicin

The K88 antigen factor The determinant of this antigen was transferred to the *Shigella* recipient from the *E coli* strain D432 (27) EMB *xyl plates* were used for re isolation Production of the K88 antigen was demonstrated in 12 per cent of the colonies by means of slide *agglutination* test performed with culture from broth agar plates *As in the case of E coli* strains the K88 antigen caused O inagglutinability in the *Shigella* strain *and this effect disappeared after boiling* for 30 minutes

Comparison of the Antigens Determined by the Different Plasmids

All the antisera produced were cross absorbed and all strains were examined in cross agglutination tests in the absorbed antisera

The *F and F13 lac factors* determine the same f *antigen since* they mutually depleted the antiserum of each other (Tables 2 and 3) None of the strains carrying the other plasmids except colB2 agglutinated in these antisera as soon as the normal *Shigella* antibodies had been removed by absorption with the plasmid negative strain

Each of the *colE1a* and *K88 factors* determines an antigen which is ...t mutually related or related to any of those tested *Sh flexneri* 4b ...lE1a agglutinated to titre 80–160 in homologous antiserum after ab...rption with the plasmid negative strain and all other plasmid ...rrying strains also failed to agglutinate in this serum and to remove ...e colE1a antibodies The picture was similar in the case of the K88 ...rain apart from the fact that the titre in homologous antiserum was ...gher (2560)

TABLE 2

Tube Agglutination Test Performed with Antiserum against D4101 (Sh flexneri 4b F)

Antigen (live culture)	Antiserum against D4101 absorbed by live culture of UP4009 (–)	D4306 (R)	D4404 (colE1a) or D4460 (K88)	D4472 (colB?)	D4451 (F13)
UP4009 (–)	0§	0	0	0	0
D4101 (F)	2560†	2560	2560	640	0
D4451 (F13)	2560	2560	2560	640	0
D4306 (R)	0	0	0	0	0
D4472 (colB2)	2560	1280	2560	0	0
D4404 (colE1a)	0	0	0	0~	0
D4460 (K88)	0	0	0	0	0

The plasmid carried F13 = F13 lac R = R100 1

§ Titres lower than 20

† Titre = reciprocal value of highest dilution of serum displaying a macroscopical reaction

TABLE 3

Tube Agglutination Test Performed with Antiserum against D4451 (Sh flexneri 4b F13)

Antigen (live culture)	Antiserum against D4451 absorbed by live culture of UP4009 (–)	D4306 (R)	D4404 (colE1a) or D4460 (K88)	D4472 (colB?)	D4101 (F)
UP4009 (–)	0§	0	0	0	0
D4101 (F)	2560†	2560	2560	640	0
D4451 (F13)	560	2560	2560	640	0
D4306 (R)	0	0	0	0	0
D4472 (colB?)	2560	2560	2560	0	0
D4404 (colE1a)	0	0	0	0	0
D4460 (K88)	0	0	0	0	0

Symbols See Table 2

The R100 1 factor The R100 1 strain D4301 was not agglutinated in any of the antisera after absorption with the plasmid negative strain In contrast the F and F13 lac strain agglutinated in the antiserum

produced with D4301 Based on other experimental observations (10) the possibility was suggested that this non agglutinability was connected with restriction of foreign plasmid DNA According to this hypothesis the same R factor was transferred to an *E coli x Sh flexneri* 4b hybrid strain D4042/18 which carried the hsp (host specificity) gene of K12 The result was that the new R100 1 derivative D4306 of this hybrid strain did agglutinate in the R100 1 anti serum of strain D4301 (Table 4) and gave a low titre in the anti colB2 serum but failed to agglutinate in the F and F13 lac antisera (Tables 2 and 3) Absorption of the R100 1 antiserum with the F and F13 lac strains did not remove the R100 1 antibodies In other words a relationship exists between the antigens determined by the F and R100 1 factors which cannot at present be finally elucidated

TABLE 4

Tube Agglutination Test Performed with Antiserum against D4301 (Sh flexneri 4b R100 1)

Antigen (live culture)	Antiserum against D4301 absorbed by live culture of			
	UP4009 (–)	D4404 (colE1a) or D4460 (K88)	D4472 (colB2)	D4101 (F) or D4451 (F13)
UP4009 (–)	0§	0	0	0
D4101 (F)	1280†	1280	80	0
D4451 (F13)	1280	640	160	0
D4306 (R)	1280	1280	320	640
D4472 (colB2)	320	320	0	0
D4404 (colE1a)	0	0	0	0
D4460 (K88)	0	0	0	0

Symbols See Table 2

TABLE 5

Tube Agglutination Test Performed with Antiserum against D4472 (Sh flexneri 4b colB2)

Antigen (live culture)	Antiserum against D4472 absorbed by live culture of			
	UP4009 (–)	D4404 (colE1a) or D4460 (K88)	D4101 (F) or D4451 (F13)	D4306 (R)
UP4009 (–)	0§	0	0	0
D4101 (F)	640†	1280	0	0
D4451 (F13)	640	640	0	0
D4306 (R)	80	80	0	0
D4472 (colB2)	2560	2560	1280	2560
D4404 (colE1a)	0	0	0	0
D4460 (K88)	0	0	0	0

Symbols See Table 2

The colB2 factor The colB2 strains agglutinated in homologous F F13 lac and R100 1 antisera after absorption by the plasmid negative

train In colB2 antiserum both the F the F13 lac and the R100 1+ strains agglutinated but none of these were capable of removing the colB2 antibodies All these strains are antigenically related The F and R100 1+ strains share a common factor 'a The colB2 strain does not possess the whole of factor a but only a part of it and in addition it has a specific factor not present in either the F+ or the R100 1 strain

TABLE 6

The Effect of K88 Infection on the Expression of Agglutinability and Sex Specific Phage Sensitivity of Strains D4101 (F) and D4404 (colE1a)

Strains	Agglutinability		Phage sensitivity (e o p)	
	Antiserum †	Antiserum M533//	f1	If1
D4101 (F)	1280§	–	5×10^{10}	–
D4101 1 (F K88)	20	–	0†	–
D4404 (colE1a)	–	160	–	4×10^{12}
D4404 1 (colE1a K88)	–	20	–	0

The plasmid carried

§ Titre = reciprocal value of highest dilution of serum displaying a macroscopical reaction

// Antiserum produced against M533 of *S typhimurium* (colE1a)

† E o p lower than 10

Sensitivity of F and I Specific Phages

The F specific phage f1 attacked the F+ F13 lac+ R100–1 and the colB2 *Sh flexneri* 4b strains while the colE1a strain was sensitive to the I specific phage If1 only and was the only one sensitive to this phage Where sensitivity was shown the e o p was about 10^{10}–10^{12} None of the two phages formed plaques on the K88 strain

Inhibitory Effect of the K88 Factor

In the *E coli* group the K88 factor exerts an inhibitory effect on the F factor (27) *i e* it belongs to the fi factors This ability of the K88 factor was shown by agglutination and phage sensitivity tests to be at work also in the *Sh flexneri* group Furthermore it was demonstrated (Table 6) that the K88 plasmid also inhibited the colE1a factor The colE1a strain agglutinated to a low titre of 160 in the *colE1a S typhimurium* antiserum but the titre was reduced to 20 after introduction of the K88 factor However the inhibition was very conspicuous as regards sensitivity to phage If1 The e o p decreased from 4×10^{1} to 0 or at any rate below 10 The presence or absence of F or colE1a factors had no influence on the percentage of *Sh flexneri* 4b cells which could receive the K88 plasmid in a mixed cultivation experiment

DISCUSSION

One of the means by which the relationship between transmissible plasmids can be assessed is serological analysis It is generally accepted that all cells possessing such plasmids also possess what is now called sex fimbriae (16) These are divided into two main groups the F like and the I like There is reason to believe that these structures can be demonstrated by the bacterial agglutination technique in the same way as the f+ antigen determined by the F factor This has been tried in the *E coli* group with R100 1 carrying strains without real success because of simultaneously acquired autoagglutinability However it was concluded that some relationship exists between the f+ antigen and the antigen determined by the R factor *Sekijima & Iseki* (22) found that *E coli* and *Salmonella* infected with the R100 1 factor developed r+ antigens which were mutually but not identically related The aim of the present investigation was to see how plasmid carrying *Sh flexneri* strains behave in this respect

It was previously reported that the f antigen in the *Shigella* group was identically related to the f antigen in the *E coli* group It has now been shown that F and F13 lac carrying *Sh flexneri* 4b strains have identical antigens

The strain infected with the R100 1 factor a derepressed mutant of the fi factor R100 did not agglutinate in any of the antisera produced not even the homologous antiserum However when the R100 1 factor was transferred to a mutant of the same *Sh flexneri* 4b strain which by recombination with K12 had received the K12 hsp gene the strain agglutinated in the antiserum of the normally produced *R100 1 Sh flexneri* 4b strain and to some degree in the colB2 antiserum

The antigen present in this strain was related to the f antigen but since only unilateral relationship was demonstrated the exact relationship could not be elucidated It was suggested that the phenomenon of non agglutinability of the first R100 1 *Sh flexneri* 4b strain produced might be explained by host specific modification but this concept needs further support

Lawn et al (13) studied sex fimbriae serologically by examining antiserum labelled culture in the electron microscope (12) They concluded that there is an antigen common to F and R fimbriae but that F fimbriae have an extra antigenic component which is not represented in RI This result cannot be compared with that presented here since they investigated *Salmonella* strains carrying the plasmid *RI*

The derepressed colB2 factor belonging to the col factors which carry the gene determining production of an F like sex fimbria was shown also to determine an antigen that is serologically related to the f antigen and to the R100 1 determined antigen without any of them being identical with one of the others

The f antigen and the *antigens determined by R* and *colB factors*

are thus serologically related also when present in a *Shigella* strain. The colE1a and K88 carrying strains reacted as expected in that they each determined their own antigens both of which were unrelated to those determined by the F like group of plasmids.

However the K88 antigen which has the same structure as fimbriae (23) can hardly belong to the sex fimbriae itself. Its determinant is probably only fortuitously linked to a sex factor (replicon) that determines the real sex fimbriae. An *E coli* strain which after transfer of the K88 factor has acquired the ability to chromosomal transfer may keep this ability even after loss of the K88 antigen and introduction of the K88 factor into an F+ strain inhibits the F character (27) i e the factor belongs to the fi class. No plaques can be found with F specific phages. *Meynell et al* (16) however count the K88 plasmid among the F like because they found an increase in titre following addition of F specific phage.

The fi character of the K88 plasmid was also at work in *Sh flexneri* 4b since the f antigen and the F phage sensitivity disappeared. The colE1a determined antigen and sensitivity to If1 phage suffered the same fate when the K88 factor was transferred to a colE1a carrying strain. The influence on the colE1a production was not examined. Before a true inhibition effect is considered it must be excluded that this latter fact can be explained by a masking effect since the K88 antigen in the *E coli* group is known to equip the bacterial cell with a thick fur like coat (23 25).

SUMMARY

The *Escherichia coli* plasmids F F13 lac R100 1 colB2 colE1a and K88 were introduced into a *Shigella flexneri* 4b. The surface antigens genetically determined by these plasmids were analysed serologically by bacterial agglutination tecnique and antisera against the strains with the different factors were cross absorbed.

Sh flexneri 4b strains carrying antigens determined by the plasmids F F13 lac R100 1 and colB2 were sensitive to the F specific phage f1. The antigens determined by F and F13 i e f+ antigen were identically related while the antigen determined by R100 1 shared a common factor a with the f antigen. The colB2 strain possessed a part of factor a and in addition had its own specific factor.

Sh flexneri 4b with the colE1a factor was sensitive to phage If1 did not agglutinate in any heterologous antiserum absorbed with the plasmid negative strain and gave only a low titre in absorbed homologous antiserum. No other plasmid carrying strain agglutinated in this antiserum.

The K88 carrying *Sh flexneri* 4b strain was insensitive to both phages f1 and If1. Serologically the K88 antigen was unrelated to the antigens determined by the other factors. Sensitivity to phage f1 or If1 disappeared after introduction of the K88 determining factor.

REFERENCES

1 *Adams M H* Methods of study of bacterial viruses Meth med Res 2 1-73 1950

2 *Brinton C C Jr* Non flagellar appendages of bacteria Nature (Lond) *183* 782-786 1959

3 *Datta N Lawn A M & Meynell E* The relationship of F type piliation and F phage sensitivity to drug resistance transfer in R F *Escherichia coli* K12 J gen Microbiol 43 365-376 1966

4 *Duguid J P Smith I W Dempster G & Edmunds P N* Non flagellar filamentous appendages (fimbriae) and haemagglutinating activity in Bacterium coli J Path Bact *70* 335-348 1955

5 *Fredericq P* Colicins Ann Rev Microbiol *11* 7-22 1957

6 *Hausmann C & Clowes R* Fertility repression by colicinogenic B factors in Escherichia coli Bact Proc GP47 1968

7 *Hirota Y Nishimura Y Orskov F & Orskov I* Effect of drug resistance factor R on the F properties of Escherichia coli J Bact 87 341-351 1964

8 *Ishibashi M* F pilus as f antigen J Bact *93* 379-389 1967

9 *Kétyi I* Acquisition of the K 12 F male state and the f antigen by Shigella flexneri strains Acta path microbiol scand 75 104-110 1969

10 *Kétyi I & Ørskov F* Host controlled modification and restriction of *foreign* chromosomal and plasmid DNA in Shigella flexneri strains In press

11 *Knolle P & Ørskov I* The identity of the f antigen and the cellular receptor for the RNA phage fr Molec Gen Genetics *99* 109-114 1967

12 *Lawn A M* Simple immunological labelling method for electron microscopy and its application to the study of filamentous appendages of bacteria Nature (Lond) *214* 1151-1152 1967

13 *Lawn A M Meynell E Meynell G G & Datta N* Sex pili and the classification of sex factors in the Enterobacteriaceae Nature (Lond) *216* 343-346 1967

14 *Lederberg J & Lederberg E M* Replica plating and indirect selection of bacterial mutants J Bact *63* 399-406 1952

15 *Loeb T & Zinder N D* A bacteriophage containing RNA Proc Nat Acad Sci (Wash) *47* 282-289 1961

16 *Meynell E Meynell G G & Datta N* Phylogenetic relationships of drug resistance factors and other transmissible bacterial plasmids Bact Rev 32 55-83 1968

17 *Meynell G G & Datta N* Functional homology of the sex factor and resistance transfer factors Nature (Lond) 207 884-885 1965

18 *Meynell G G & Datta N* The relation of resistance transfer factors to *the F* factor of Escherichia coli K12 Genet Res 7 134-140 1966

19 *Meynell G G & Lawn A M* The sex factor of colicin factor Ela *Genet Res* *10* 323-325 1967

20 *Meynell G G & Lawn A M* Filamentous phages specific for the I sex factor Nature (Lond) *217* 1184 1186 1968

21 *Nishimura Y Ishibashi M Meynell E & Hirota Y* Specific piliation *directed by* a fertility factor and a resistance factor of *Escherichia coli* J gen Microbiol 49 89-98 1967

22 *Sekijima Y & Isehi S* On the r+ antigens of bacteria having *drug resistance* transfer factor R Proc Jap Acad *42* 984-986 1966

23 *Stirm S Ørskov F Ørskov I & Birch Andersen A* Episome carried surface antigen K88 of Escherichia coli J Bact *93* 740-748 1967

24 *Zinder N P Valentine R C Roger M & Stoeckenius W* f1 a rod shaped male specific bacteriophage that contains DNA Virology 20 638 640 1963

25 *Ørskov F & Ørskov I* Staining of Escherichia coli K88 antigen and other fimbrial antigens for light microscopy *J gen Microbiol Proceedings* of the Edinburgh Meeting of the Society Sept 1968 53 XXI 1969

26 *Ørskov I & Ørskov F* An antigen termed f in F E coli strains Acta path microbiol scand *48* 37-46 1960

27 *Ørskov I & Ørskov F* Episome carried surface antigen K88 of Escherichia coli I Transmission of the determinant of *K88 antigen* and influence on the transfer of chromosomal markers *J Bact 91* 69-75 1966

Acta path microbiol scand 77 309-317 1969

Department of Virology and Department of Serology and Bacteriology
University of Helsinki and Finnish Red Cross Blood Transfusion Service Helsinki
Finland

SOLUBLE ANTIGEN ANTIBODY COMPLEXES AND PLATELET AGGREGATION

By

K. Penttinen G. Myllylä O. Mäkelä and A. Vaheri

Received 16 iv 69

The platelet aggregation (PA) test based on the sedimentation pattern of platelets on microplates can be used to demonstrate direct interaction of myxo and reoviruses with platelets (11 12) It can also detect platelet aggregation induced by soluble antigen antibody complexes (11 12) In detecting rubella antibodies the PA method was as sensitive as haemagglutination inhibition and more sensitive than complement fixation (CF) (6) In cases of postrubella thrombocytopenic purpura the PA antibody titres were exceptionally high In the case of cytomegalovirus antibodies the PA test gave up to ten times higher titres than the CF test and needed less than one tenth as much antigen (5) The PA test can also detect small amounts of arbo B virus antigens and antibodies (13) However the CF test with crude antigen was more effective than the PA test in detecting serum antibodies in patients with acute herpes varicella and measles infections when preparations of small size antigen were used (10) Some virus antigen preparations with their antibodies do not seem to aggregate platelets (12)

Under our test conditions it seems clear that the platelets are aggregated by antigen antibody complexes (7) Consequently PA reactivity can be affected by variations both in antigen and in antibody With viral antigens and antibodies the system is complex because each virus antigen used has probably many different determinants and there are antibodies of many different specificities To simplify the system we used bovine serum albumin (BSA) and anti BSA antibodies for some experiments The PA technique seemed to work well with these reagents and there was platelet aggregation in both higher and lower antigen dilutions and higher serum dilutions than the visible precipi

---

Request for reprints should be addressed to *K. Penttinen* Department of Virology University of Helsinki Haartmaninkatu 3 Helsinki 29 Finland

Aided by grants from the *Sigrid Jusélius Foundation* and the *Finnish Research Council for Medical Sciences*

tates However even in the BSA anti BSA system the antigenic determinants are complex and there are multiple sets of antigens and we wanted even simpler conditions Consequently we have used as antigen the synthetic determinant 4 hydroxy 3 iodo 5 nitro phenyl acetic acid (NIP) (9) and monospecific rabbit serum against NIP hapten NIP was coupled to BSA or human serum albumin (HSA) *in different molar* ratios (from 1 2 to 40) and we compared the PA and CF reactions and made some sucrose gradient analyses

MATERIALS AND METHODS

The antigens NIP_1 BSA NIP BSA NIP_{0}HSA NIP_{4}BSA and NIP_{40}BSA were prepared as described earlier (2) The number of NIP groups coupled to each protein molecule was estimated by *determining the optical density of the conjugates at* 430 nm The anti NIP serum (code 2037) was prepared by immunizing a rabbit with alum precipitated NIP_1 chicken globulin It received 3 injections of 1 mg each at 6–8 week intervals and was bled 10 days after the third injection Quantitative precipitation with NIP_{40}BSA showed that the antiserum contained 0 42 mg/ml of anti NIP antibody

Platelet aggregation test The technique has been described earlier (11 6) In brief a washed human platelet suspension was prepared by differential centrifugation (1) and the platelets were finally suspended (200 000 platelets/mm^3) in a slightly alkaline medium pH 7 8 The platelets were used the same day they *were prepared Antigen diluted in the same medium (0 05 ml) and diluted anti* serum (0 05 ml) were mixed in U microplates After one hour at room temperature the platelet suspensions (0 05) were added Only checkerboard titrations were used in this work Antigen and serum controls in all dilutions used were included The plates were incubated overnight at a temperature of + 5 to + *8* C and *the* sedimentation patterns were read the following morning with dark background illumination The factors affecting the platelet aggregation reaction will be described separately (7 8)

Complement fixation reaction The same microplates were used for the CF reaction 0 025 ml of diluted antigen and 0 025 ml of diluted antiserum were *incubated overnight at + 4 C* with 0 025 *ml of complement (stock of routinely* used guinea *pig serum)* The following morning the haemolytic system was added The antigens were not anticomplementary in the dilutions used The anti NIP serum showed some anticomplementary activity in dilutions 20 and 40 (reciprocal values) This was compensated for by calculated additional amounts of complement in these two serum dilutions In the series shown in Table 3 *slightly less than* 2 full units of complement were used

Gradient centrifugation A sample of 0 2 ml was layered *on 4 5 ml of a linear* 15 5 to 33 per cent (w/vol) sucrose gradient in *0 022 M Tris Cl* buffer (ionic strenght 0 04 M) pH 7 5 and was centrifuged at + 4 C in the SW 50 rotor of a *Spinco centrifuge The fractions* were collected from *a hole in the* bottom of the tube In some experiments a SW 27 rotor with a sample volume of 1 ml and 34 ml of gradient was used Paul Bunnell positive *serum* was *used a* a reference marker The sucrose in Tris buffer did not affect the platelet settling pattern above dilution 8 of the fractions

RESULTS

PA Reaction

We did some preliminary titrations to find which antibody/antigen combinations gave positive *results (Fig 1) The first dilution* of anti NIP was 20 Under the same conditions the number of positive antibody/antigen combinations varied with *different* platelet preparations With seven different lots of platelets and NIP ,BSA as antigen the

TABLE 1

The Effect of Hapten Density on the Platelet Aggregation (PA) Test Using Monospecific Antiserum

Moles of NIP per mole of carrier	Moles of Ab per moles of conjugate		Concentration of antigen†				
			Moles/l of BSA		Moles/l of NIP		g/ml
	First positive§	Last positive§	First positive	Last positive	First positive	Last positive	Last positive
1 2	–	–	–	>2 7×10^{-6}	–	>3 1×10^{-6}	>180×10^{-6}
2	0 5	4	50×10^{-9}	6 3×10^{-9}	105×10^{-9}	13×10^{-9}	420×10^{-9}
10	0 5	60	13×10^{-9}	98×10^{-12}	125×10^{-9}	1 0×10^{-9}	6 5×10^{-9}
24	0 2	50	13×10^{-9}	49×10^{-12}	315×10^{-9}	1 2×10^{-9}	3 3×10^{-9}
40	0 1	60	13×10^{-9}	24×10^{-12}	520×10^{-9}	1 0×10^{-9}	1 6×10^{-9}

HSA instead of BSA

§ First and last positive Ab/Ag combinations have been calculated from the serum dilution 1/160 giving the highest titre for antigen

† The values represent initial dilutions of reagents

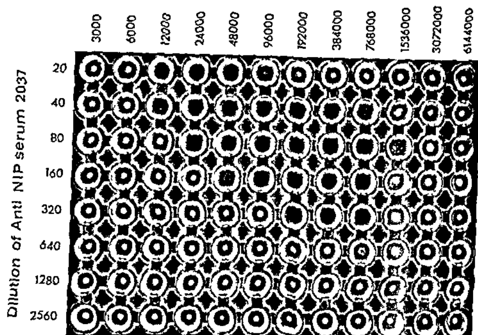


Fig 1

Patterns of platelet aggregation by antigen antibody complexes (Anti NIP serum and $NIP_{10}BSA$ antigen)

TABLE 2

The Effect of Hapten Density on the Platelet Aggregation (PA) Test Using Monospecific Antiserum

Moles of NIP per mole of carrier	Moles of Ab per moles of conjugate		Titre of serum		Number of positive Ab/Ag combinations
	First *positive*	Last *positive*	G of Ab/ml	Dilution	
1 2	–	–	$>21\times10^{6}$	<20	0
2	0 5	4	$1\,3\times10^{6}$	320	18
10§	0 5	60	$0\,7\times10^{6}$	640	41
24	0 ?	50	$0\,3\times10^{-6}$	1280	56
40	0 1	60	$0\,3\times10^{6}$	1280	59

§ See explanations in Table 1

numbers of positive results were 43 45 46 48 49 55 and 56 We have found however that 10–15 per cent of the individual platelet preparations are almost nonreactive with soluble immune complexes The results of titrating the different antigens with the most sensitive platelet preparation are shown in Tables 1 and 2

$NIP_{1\,2}BSA$ antigen never produced platelet aggregation Even in experiments beginnin from 1 1 per cent concentration of $NIP_{1\,2}BSA$

and dilution 5 of anti NIP serum the PA was negative With all other antigens the first positive reaction was at an antibody/antigen ratio (calculated from the precipitation data) of 0 5-0 1 Thus the number of NIP molecules on each carrier molecule had some effect However increasing the hapten density from 2 to 10 caused the highest positive antibody/antigen ratio to increase from 4 to 60 It could not be increased further by using NIP ,BSA or $NIP_{40}BSA$ The antigen sensitivity of the PA test increased when the coupling ratio increased the smallest amount of antigen that could be detected decreased from 420 to 1 6 nanograms/ml The titre of the anti NIP serum increased with increasing hapten density only from 320 to 1280

CF Reaction

The corresponding results obtained with the CF reaction are shown in Table 3

TABLE 3

The Effect of Hapten Density on the CF Reaction with Monospecific Antiserum

oles of IP per ole of arrier	Moles of Ab per moles of conjugate		G of antigen /ml	Titre of serum		Number of positive Ab/Ag combinations
	First positive	Last positive	Last positive	G of Ab/ml Last positive	Dilution	
1 2	–	–	$>11\times10^{3}$	$>21\times10^{6}$	<20	0
	–	–	$>12\times10^{3}$	$>21\times10^{6}$	<20	0
10§	0 25	?0	16×10^{7}	11×10^{-6}	40	6
24	0 15	2 5	$3\ 1\times10^{7}$	$5\ 3\times10^{-6}$	80	11
40	0 3	2 5	$1\ 6\times10^{7}$	$5\ 3\times10^{6}$	80	9

§ See explanations in Table 1

$NIP_{1\ 2}BSA$ and NIP BSA had no demonstrable activity in the CF test Using other antigens positive antibody/antigen combinations were 5–7 times fewer than in the PA test The sensitivity of the CF test was less than that of the PA test both as regards the detection of antigen (100 times less) and the detection of antibody (16 times less)

Gradient Centrifugation of the Antigen Antibody Complex

In sucrose gradient analysis the NIP_2 BSA antigen sedimented at the expected rate (the carrier BSA molecule has a sedimentation coefficient of about 4 5 S and a molecular weight of 67000) The position of the antigen was determined either by the PA test (with added antiserum) or by optical adsorbance at 430 nm The anti NIP antibody was in the 7S region A mixture of $NIP_{24}BSA$ antigen and anti NIP serum at antibody/antigen ratios of 0 2–1 produced soluble antigen antibody complexes in the 20–25 S regions These could be detected by platelet aggre

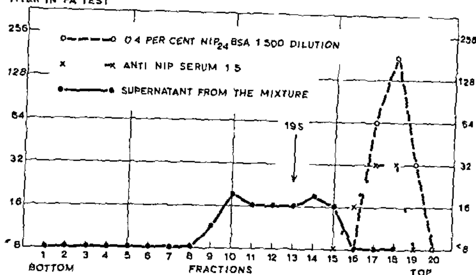


Fig 2

Velocity gradient centrifugations of 0.4 per cent $NIP_{24}BSA$ 1:500 dilution, anti NIP serum 1:5 and the supernatant from a mixture obtained by adding $NIP_{24}BSA$ 1:500 to anti NIP serum 1:5. Incubation at room temperature for 90 min, centrifugation at 3000 g for 30 min. Linear sucrose gradient 15.5–33 per cent w/vol in 0.09 m tris buffer pH 7.5. SW 27 rotor 25 000 RPM 14 hours at + 3 C.

gation without added antigen or antibody (so called *direct PA activity*) as shown in Fig 2. In addition we found that the PA activity of the immune complexes in the 20–25 S fractions was blocked by added antigen and unaffected or slightly enhanced by added antibody.

DISCUSSION

It is interesting that the reactivity of the antigen increased with increasing hapten density. This has been shown previously for the CF reaction (3) and we found it to be true of both CF and PA. In neither test did we get any reaction with NIP_1 BSA. Changing the hapten density from 2 to 10 caused a marked change in the CF test from negative to clear positive and in the PA test the lowest amount of antigen detectable increased 64 times in terms of BSA molarity and 13 times in terms of NIP molarity. A further increase in hapten density did not produce any essential change. In the PA test the antibody titre only increased four fold with increasing hapten density.

The fact that an increase in the average coupling ratio from 2 to 10 greatly increased the effectiveness of our antigens in the PA test suggests that the density of hapten groups on the surface of the carrier molecule is important. The formation of complexes that are both stable and soluble may require that both combining sites of an antibody molecule are attached to an antigen molecule. Another requirement

might be that more than one antibody molecule must be attached to a single antigen molecule The sedimentation rates of the complexes may suggest that several antibody molecules are attached to most of the antigen molecules The different reactivity of NIP BSA in the two tests may be due to the greater sensitivity of the PA test or to different spatial requirements for hapten localization on the carrier molecule

When antigen and antibody are mixed it is likely that immune complexes of various sizes are produced However the soluble complexes that can aggregate platelets both in rubella (14) and in the NIP anti NIP system have sedimentation rates of about 20–25S The finding that added antigen or antibody changes the PA activity of the 20–25S particles strongly suggests their specific immune complex nature

From the results it appears that in cases of high antibody excess sensitive platelets can be aggregated by 100 or less NIP_{40}BSA molecules per platelet Soluble antigen antibody complexes induce viscous metamorphosis and subsequent aggregation of platelets The exact nature of the aggregation is uncertain but it is a response of platelets to the interaction of immune complexes with the platelet surface and depends on the metabolic state of the platelets (8) Thus it differs from the usual serological agglutination mechanisms

In all our studies the antibodies that could give positive PA reactions have been 7S but it is not certain that other classes of antibodies are ineffective Our platelet aggregation does not seem to require heat labile components of complement (7)

The results of the PA test depends on the particular batch of platelets used The number of positive antibody antigen combinations with the same reagents and with different lots of platelets may be used as an indicator of the tuning i e the readiness of the platelets to react With less sensitive platelets the positive area decreased from all sides

We used the routine serological technique for the CF test With some virus antigens we found that the titre of positive sera in the PA test could be more than 10 times higher than in the CF test (6 5 13) With monospecific antiserum and various different NIP carrier conjugates there was an 16 fold difference in titre The sensitivity of the PA technique is shown by its ability to detect 0 3 µg/ml of anti NIP antibody while the CF test used cannot detect less than 5 3 µg/ml With various antigens we found that the PA test required less antigen than the CF test (6 5 13 10) The smallest amount of NIP_{40}BSA we could detect with the PA test was 1 6 ng/ml with the CF test 160 ng/ml

The present experiments concerned only one set of antibodies and one antigenic determinant and both the PA and CF reactions were quantitative The prozone was very marked in both reactions and the optimum quantity of antigen for measuring antibody was limited to a narrow zone In the PA test the optimum antibody dilutions for detecting antigen were 80 and 160 higher antibody concentrations inhibited the reaction (Fig 1) This may be because the antibody/antigen

ratio is inappropriate in high antibody concentrations or because the antiserum contains nonspecific factors that can interfere with platelet aggregation when present in sufficiently large amounts In the case of virus antigens the prozone was not so clear although it could usually be seen in the PA test (13) This lack of prozone was probably due to the overlapping areas of positive antibody/antigen combinations when multiple sets of antigens and antibodies were involved We have studied the PA test in a number of virus diseases In some it detected antibodies more effectively than did the CF test in others the reverse was true (see introduction) Many viral structures contain identical repeating units The behaviour of a virus antigen in the PA test may depend on the frequency with which its antigenic determinants are repeated Virus antigens with certain determinant densities may cause damage to platelets also *in vivo* (6 13) if antigen production continues during the period of antibody excess We have shown that the complexes are active in antibody excess Enhancement of activity of antigen antibody complexes in antibody excess has also been observed by the histamin release technique (4) This activity in antibody excess is necessary if the complexes are to act during convalescence when the free antibody is present

SUMMARY

A micromethod involving platelet sedimentation patterns was used to determine antibodies and antigens in virus diseases Platelet aggregation (PA) appears to be caused by the reaction of soluble antigen antibody complexes with platelets The reaction was further investigated with the antigenic determinant NIP coupled to BSA or HSA The molar ratio NIP BSA was 1 2 2 10 24 or 40 in different preparations Anti NIP serum produced no PA with $NIP_{1\,2}$BSA but all the other antigens induced PA from an Ab/Ag ratio of 0 5–0 1 upwards NIP_2 antigen produced PA at Ab/Ag = 4 and the others at Ab/Ag = 50–60 With increasing amounts of NIP per mole albumin the antibody titre increased from 320 to 1280 The smallest amount of detectable antigen was 1 6 *ng/ml* of NIP_{40}BSA antigen Immune complexes active in PA sedimenting at 20–25 S were demonstrated by gradient centrifugation The PA reaction was more sensitive than the CF test NIP_1 BSA and NIP BSA were not active in CF test when the first tested serum dilution was the same (20) as in PA experiments The smallest amount of NIP_{40}BSA antigen detectable was 0 16 μg/ml The titre of Anti NIP serum in CF test was 80 With PA technique 0 3 μg/ml and with CF technique 5 3 μg/ml anti NIP antibody was measurable The results support the hypothesis that the effective antigens contain at least two functional groups and that PA active soluble immune complexes are formed also in antibody excess The possible significance of the findings with regard to viral infections is discussed

REFERENCES

1 *Aster R H & Jandl J H* Platelet sequestration in Man I Methods J Clin Inv 43 843–855 1964
2 *Brownstone A Mitchison N A & Pitt Rivers R* Chemical and serological studies with an Iodine-containing synthetic immunological determinant 4 hydroxy 3 iodo 5 nitro phenylacetic acid (NIP) and related compounds Immunology 10 465–479 1966
3 *Davis B D Dulbecco R Eisen H N Ginsberg H S & Wood W B* Microbiology Hoeber Medical Division Harper and Row Publishers New York Evanston and London 1968 p 514
4 *Goele D J & Osler A G* In vitro damage of rabbit platelets by an unrelated antigen antibody reaction I General characteristics of the reaction J Immunol 94 236–246 1965
5 *Penttinen K Kääriäinen L & Myllylä G* Cytomegalovirus antibody assay by platelet aggregation To be published in Arch ges Virusforsch
6 *Myllyla G Vaheri A Vesikari T & Penttinen K* Interaction between human blood platelets viruses and antibodies IV Post rubella thrombocytopenic purpura and platelet aggregation by rubella antigen antibody interaction Clin exp Immunol 4 323–333 1969
7 *Myllylä G & Penttinen K* Interaction between human blood platelets viruses and antibodies III The sequence of the reactions and the role of complement in the aggregation of platelets by antigen antibody interaction To be published
8 *Myllylä G* Aggregation of human blood platelets by immune complexes To be published
9 *Makela O Cross A M & Ruoslahti E* Similarities between the cellular receptor antibody and the secreted antibody Biological Recognition Process R T Smith and R A Good (eds) University of Florida Press In Press
10 *Palosuo T Penttinen K & Myllyla G* Platelet aggregation by Herpes Simplex antigen antibody complexes To be published
11 *Penttinen K & Myllyla G* Interaction of human blood platelets viruses and antibodies I Platelet aggregation test with microequipment Ann Med exp Fenn 46 188–192 1968
12 *Penttinen K & Myllyla G* Interaction between human blood platelets viruses and antibodies II Interaction of different viruses with platelets The effect of antibodies To be published
13 *Penttinen K Saikku P Myllylä G Brummer Korvenkontio M & Oker Blom N* The platelet aggregation test in group B arbovirus infections To be published in J Hyg 1970
14 *Vaheri A Vesikari T Penttinen K & Myllylä G* Soluble rubella antigens platelet aggregation and postrubella thrombocytopenia 23rd Symposium on Microbiological Standardisation Rubella Vaccines London 1968 Symposia Series in Immunobiological Standardization 11 107–108 Ed Karger Basle/New York 1969

Acta path microbiol scand 77 318-324 1969

The Finsen Laboratory The Finsen Institute *Copenhagen Denmark*

THE MECHANISM OF ERYTHROCYTE DESTRUCTION INDUCED BY INJECTION OF BACTERIAL PYROGEN

By

HANS KARLE

Received 5 xi 69

An increased erythroclasia has been demonstrated in rabbits in relation to experimental fever (*Karle* 1968a) The elevated body temperature was in part produced by intravenous (i v) injection of bacterial pyrogen A quantitative evaluation revealed a certain correlation between the fever response and the degree of red cell destruction while the amount of pyrogen was found to be without influence (*Karle* 1969b) It raises the question therefore whether the pyrogen provokes the destruction of the erythrocytes as heat damage through the elevation of body temperature or as a result of a direct action upon the red cells or cells in the reticuloendothelial system (R E S)

To obtain this differentiation the survival of erythrocytes was examined in rabbits receiving pyrogen under simultaneous suppression of the fever response

MATERIAL AND METHODS

Rabbits weighing 2000-4000 g were used As pyrogen a suspension of killed bacteria from a member of the Faecalis alcaligenes group an apatogen microbe related to *E coli* was used The suspension contained 10^9 bacteria per ml (Vaccine for fever therapy Statens Seruminstitut Copenhagen) The pyrogen was administered by i v injection Details concerning injections blood sampling and determination of ordinary haematological parameters have been mentioned previously (*Karle* 1968a)

Recording of rectal temperature was made by an Flectro Universal Thermometer (Ellab Instruments Copenhagen kindly lent us by The Control Laboratories of the Danish Pharmacist Association) which permitted simultaneous measurements in 10 rabbits

Fever response was expressed as Fever Index (Δ degree C hours and determined by weighing out the area below the fever curve

Labelling of autologous erythrocytes with $Na_2{}^{51}CrO_4$ was made as previously mentioned (*Karle* 1968a) In one part of the study ^{59}Fe labelled erythrocytes from a donor rabbit were used The donor was labelled with about 20 µCi ^{59}Fe citrate 40 days before the study and received 50 mg Fe^{++} 2-3 times weekly starting five days after the injection of tracer in order to prevent reutilization of marker from the physiological breakdown of red cells

---

Study performed during the tenure of research fellowship from *The Danish Anti Cancer League*

All measurements of radioactivity were made in a well scintillation counter In ^{51}Cr studies the activities were expressed per ml blood in percentage of the value of the first sample which was taken about 15 min after reinjection of labelled erythrocytes ^{59}Fe activity in the blood from recipients was expressed in counts per min per ml blood The loss of ^{59}Fe in the circulation in the first period after the transfusion was calculated by the method of least squares using the logarithm of counting results from the first seven post transfusion days The coefficient of variation in the ^{51}Cr method was 2 5 per cent and in ^{59}Fe measurements 3 per cent

Before cross transfusion, the erythrocytes were washed once with NaCl 0 9 per cent and compatibility between the recipients and the donor erythrocytes was assured.

Suppression of elevation of the body temperature from injections of bacterial pyrogen was induced by 1) intramuscular (i m) injection of cortisone acetate 25 mg per day or 2) by development of pyrogen tolerance by repetitive injections of the employed pyrogen before the tracer study was performed Attempting to use phenazon it was found that doses necessary to suppress fever in themselves reduce the survival of ^{51}Cr labelled erythrocytes

RESULTS

The Effect of Cortisone upon the Destruction of Erythrocytes after Administration of Pyrogen

The principle in the investigation has been described earlier (*Karle* 1968a) After labelling of autologous erythrocytes with $Na_2{}^{51}CrO_4$ the spontaneous pattern of survival was studied by taking samples every 2 or 3 days during the first 10–12 days The fever was introduced in a five day period by daily injections of 1 0 ml pyrogen i v and the destruction of erythrocytes was followed by determining at short intervals the radioactivity the packed cell volume (PCV) the haemoglobin conc and the percentage reticulocytes Studies following this scheme were made in two rabbits which served as controls Three other rabbits were treated in the same way but from the 4th day after the labelling and until the cessation of the administration of pyrogen they received injections of cortisone acetate 25 mg per day i m In all animals the rectal temperature was recorded every 15 min in the days of pyrogen injections and the Fever Index determined as described

The rabbits which received pyrogen without cortisone responded with a distinct increase in body temperature and showed the same results as described previously (*Karle* 1968a) During the pyrogen induced fever there was a steeper slope in the survival curve and simultaneously a reduction in PCV A similar fall was demonstrated in the haemoglobin concentration After termination of injection of pyrogen the ^{51}Cr activity remained at a lower level than before the fever period although the PCV returned to normal during a period of reticulocytosis (Fig 1a) In the studies with injections of cortisone and pyrogen simultaneously the fever responses were definitely lower showing Fever Indices of 10 11 and 25 respectively compared with 60 and 70 in the experiments without cortisone and quite a different pattern in the haematological parameters were observed A represen

Acta path microbiol scand 77 318-324 1969

The Finsen Laboratory The Finsen Institute Copenhagen Denmark

THE MECHANISM OF ERYTHROCYTE DESTRUCTION INDUCED BY INJECTION OF BACTERIAL PYROGEN

By

HANS KARLE

Received 6 vi 69

An increased erythroclasia has been demonstrated in rabbits in relation to experimental fever (*Karle* 1968a) The elevated body temperature was in part produced by intravenous (i v) injection of bacterial pyrogen A quantitative evaluation revealed a certain correlation between the fever response and the degree of red cell destruction while the amount of pyrogen was found to be without influence (*Karle* 1969b) It raises the question therefore whether the pyrogen provokes the destruction of the erythrocytes as heat damage through the elevation of body temperature or as a result of a direct action upon the red cells or cells in the reticuloendothelial system (R E S)

To obtain this differentiation the survival of erythrocytes was examined in rabbits receiving pyrogen under simultaneous suppression of the fever response

MATERIAL AND METHODS

Rabbits weighing 2500–4000 g were used As pyrogen a suspension of killed bacteria from a member of the Faecalis alcaligenes group an apatogen microbe related to *E coli* was used The suspension contained 10^9 bacteria per ml (Vaccine for fever therapy Statens Seruminstitut Copenhagen) The pyrogen was administered by i v injection Details concerning injections blood sampling and determination of ordinary haematological parameters have been mentioned previously (*Karle* 1968a)

Recording of rectal temperature was made by an Electro Universal Thermometer (Ellab Instrument Copenhagen kindly lent us by The Control Laboratories of the Danish Pharmacist Association) which permitted simultaneous measurements in 10 rabbits

Fever response was expressed as Fever Index (Δ degree C hours) and determined by weighing out the area below the fever curve

Labelling of autologous erythrocytes with Na $^{51}CrO_4$ was made as previously mentioned (*Karle* 1968a) In one part of the study ^{59}Fe labelled erythrocytes from a donor rabbit were used The donor was labelled with about 20 μCi ^{59}Fe *citrate* 40 days before the study and received 50 mg Fe^{++} 2-3 times weekly starting five days after the injection of tracer in order to prevent reutilization of marker from the physiological breakdown of red cells

---

Study performed during the tenure of research fellowship from *The Danish Anti Cancer League*

In these experiments erythrocytes from a donor rabbit which received 20 µCi ^{59}Fe citrate 40 days before the study were used. When cross transfusions were made the labelled erythrocytes were relatively old and the radioactivity therefore represented erythrocytes which are sensible to injections of pyrogen (*Karle* 1968c).

Each cross transfusion constituted about 8 ml of erythrocyte suspension and were performed in three groups of animals:

A) three rabbits which received daily injections of 1 ml of pyrogen a period from 12 days before to 5 days after the transfusion.

B) two rabbits which only received pyrogen in the five days follow g the transfusion.

C) two rabbits which received no injections of pyrogen at all.

As expected some elevation of the body temperature was observed the pyrogen tolerant group (group A) but the Fever Indices were finitely lower than in group B (Table 1). After the cross transfu ons the survival of the ^{59}Fe labelled erythrocytes was followed in the cipients. In the pyrogen tolerant group the slope of the survival rves of donor erythrocytes during the continuous injections of vrogen was less steep than in the recipients which had not been xposed to pyrogen before the transfusion and thus showed more ronounced fever reactions. The slopes were somewhat steeper in the yrogen tolerant than in the untreated rabbits (group C). Table 1.

TABLE 1

Survival of ^{59}Fe Labelled Erythrocytes from Donor Rabbit during Injections of Endotoxin in Recipients with and without Pyrogen Tolerance

Animal	Type of study	Fever Index	Slope of urvival curve	Relative weight of spleen	Relative weight of liver
49 B	T	20	−0.0125	0.75	4.07
50 B	T	16	−0.0140	0.57	4.50
51 B	T	14	−0.0114	0.53	5.10
mean		16.7	−0.0126	0.61	4.56
52 B	F	38	−0.0221	1.07	4.08
55 B	F	40	−0.0276	0.55	3.72
mean		39	−0.0249	0.81	3.90
53 B	C		−0.0083	0.33	4.60
54 B	C		−0.0075	0.55	4.80
mean			−0.0079	0.44	4.70

T = pyrogen tolerance
I = without pyrogen tolerance
C = without injection of endotoxin
Concerning calculation see text

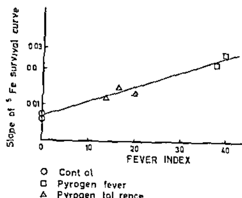


Fig 2

demonstrates the coefficients of the slopes determined for the first seven days after the transfusion and the relationship between fever response and slope of the survival curves is presented in Fig 2 Table 1 gives also the relative weights of spleen and liver at autopsy on the 10th day after the transfusion The spleens were larger in the fever group than in the pyrogen tolerant group but the differences were not significant The liver weigths showed no differences

DISCUSSION

Identical patterns of destruction of erythrocytes have previously been demonstrated in rabbits with experimental fever whether it was caused by bacterial pyrogens by injection of heated milk or by placing the rabbit in a heated chamber (*Karle* 1968a 1969b) Thus the erythrocyte destruction might well be a result of the elevated body temperature *In vitro* studies showed diminished viability of erythrocytes incubated at temperatures within the fever range (*Karle* 1969d)

In the case of the bacterial pyrogen the destruction of erythrocytes could be due to either a direct damage or be secondary to a stimulation of R E S From haemagglutination studies in vitro it is known that endotoxins of lipopolysaccharide structure from different bacteria are attachable to erythrocytes especially when the endotoxin has been treated with heat or alkali (*Neter* 1965 *Davies et al* 1958) It is however generally accepted that endotoxin will not induce haemolysis in vivo unless antibodies against the endotoxin are present (*Neter* 1965 *Shumway et al 1963)* Furthermore it has been shown that injected ^{51}Cr labelled *E coli* endotoxin fails to become *attached to* erythrocytes (*Braude Carey & Zalesky* 1955)

Ho & Kass (1958) demonstrated a haemolysis in rabbits after injection of *Salmonella typhi* endotoxin haemolysis was greatest *in immunized* animals the drop in haemoglobin concentration and the change in the ^{51}Cr survival curves did however not occur *until* 8-12

days after the first injection of endotoxin There were no demonstrable changes in osmotic and mechanical fragility of the erythrocytes and the loss was attributed to an activation of R E S stimulated by the injection of bacterial pyrogen (*Biozzi, Benacerraf & Halpern* 1953) *Keiderling* (1958) in an attempt to imitate a state of infection found a decrease in the survival in ^{51}Cr labelled erythrocytes following injections of lipopolysaccharide None of these studies however give information about body temperature

The pattern of blood changes induced by the pyrogen in this study was quite different from the findings by *Ho & Kass* The loss of erythrocytes illustrated by a decrease in PCV and haemoglobin concentration and a steeper survival curve began immediately after the onset of the fever The differences in results could be due to differences in the pyrogens used and to the immunological reactions in the case of *Ho & Kass* salmonella preparation

When corticosteroid was used as an antipyretic the effect of pyrogen on the loss of erythrocytes was significantly reduced This was confirmed by the absence of reticulocytosis during the post febrile period in which haemoglobin concentrations were restored The erythrocyte preserving action of cortisone could possibly be due *to effects other* than the suppression of fever response

The suggestion that erythrocyte destruction was related to the fever response and not to other mechanisms was further substantiated by the tolerance experiments Repetitive injections of bacterial pyrogen reduced the fever response this effect was attributed to an enhanced phagocytic activity of R E S (*Beeson* 1947) This was certainly *not* directed towards the erythrocytes since transfused red cells survived exceptionally well in tolerant animals In view of this it would appear that the lack of erythrocyte destruction in pyrogen treated splenectomized rabbits (*Karle 1968e*) could be attributed to the absence of a substantial portion of R E S capable of sequestring and destroying fever damaged erythrocytes

SUMMARY

The fever response to bacterial pyrogen has previously been shown to be accompanied by a destruction of erythrocytes the loss of red cells was correlated with the elevation of temperature The present work shows that the destruction of labelled erythrocytes is reduced whether the fever response to bacterial pyrogen is suppressed by administration of cortisone acetate or by induction of pyrogen tolerance

REFERENCES

Beeson P B Tolerance to Bacterial Pyrogens II Role of the Reticulo Endothelial System J exp Med 86 39-44 1947

Biozzi G Benacerraf B & Halpern B N The effect of Salm Typhi and its Endotoxin on the Phagocytic Activity of the Reticulo Endothelial System in Mice Brit J exp Path 36 226–235 1955

Braude A I Carey F J & Zalesky M Studies with Radioactive Endotoxin II Correlation of physiologic effects with distribution of Radioactivity in Rabbits injected with lethal doses of E Coli Endotoxin labelled with Radioactive Sodium Chromate J Clin Invest 34 858–866 1955

Davies D A L Crumpton M J Macpherson I A & Hutchinson A M The Adsorption of Bacterial Polysaccharides by Erythrocytes Immunology 2 157–171 1958

Ho M & Kass E H Hemolytic Anemia in Rabbits following Injection of Bacterial Endotoxin Proc Soc exp Biol (N Y) 97 505–509 1958

Karle H Elevated Body Temperature and the Survival of Red Blood Cells Acta med scand *183* 587–592 *1968a*

Karle H Destruction of Erythrocytes during Experimental Fever Quantitative aspects Acta med scand In press 1969b

Karle H Significance of Red Cell Age to Red Cell Destruction during Experimental Pyrexia Brit J Haemat *15* 221–229 1968c

Karle H Effect on Red Cells of a Small Rise in Temperature In Vitro Studies Brit J Haemat *16* 409–419 1969d

Karle H The Site of Abnormal Erythrocyte Destruction during Experimental Fever Brit J Haemat *15* 475–486 1968e

Keiderling W Über die Ferrokinetik beim Infect Schweiz med Wschr *88* 965–969 *1958*

Neter E Bacterial Hemagglutination and Hemolysis Bact Rev 20 166–188 1956

Shumway C N Bokkenheuser V Pollack D & Neter E Survival in immune and nonimmune Rabbits of ^{51}Cr labelled Erythrocytes modified by Bacterial Antigen N Y J Lab clin Med 62 600–610 1963

Acta path microbiol scand 77 325–3?6 1969

BRIEF REPORT

THE TUMORIGENIC EFFECT OF ETHANOL

By *Frej Stenbäck*

A number of studies conducted during the past half a century have suggested that cancer of the gastro intestinal system is associated with excessive consumption of [alc]ohol (*Gsell & Loffler* 1962 *Kuratsune et al* 1965 *Horie et al* 1965) Topical ap[pli]cation of substances to test their possible carcinogenic effect is a method in [gen]eral use not previously used in this connection

The test animals used were female mice NMRI strain 2?0 altogether divided [in]to 7 groups The chemicals used were 9 10 dimethyl 1 2 benzanthracene (DMBA) [(K]urr) acetone (chemically purified) ethanol (C_2H_5OH) 99 5 per cent concentra[tio]n and Tween 60 (polyoxysorbitolemonostearate) (Atlas) DMBA was dissolved [in] acetone (group 4) or ethanol (group 7) to make a 1 per cent solution v/w Other [su]bstances were applied as such on the back skin with a precision pipette 3 times [a] week for 20 weeks the volume being 0 02 cc The experimental groups are pre[se]nted in Table 1 The animals were kept in plastic cages 10 in each and fed ani[m]al fodder and water *ad libitum* Specimens were taken in formalin for histological [ex]amination 14 different stains were performed and the activity of 9 enzymes was [d]etermined

[R]esults

No histological or histochemical changes which could be interpreted as neoplastic [o]r preneoplastic were found in groups 1 2 5 or 6 The tumours found in group 3 [w]ere papillomas both fibropapillomas and acanthopapillomas in groups 4 and 7 [s]quamous cell carcinomas were also found The tumours in groups 4 and 7 were [h]istologically and histochemically similar The number of tumours tumour bear[i]ng animals and time of latency is shown in Table 1

TABLE 1

Group	Number of animals	*Carcinogen*	Solvent	Time of latency in weeks	Number of tumours	Number of tumour bearing animals
1	100	no	no	–	–	–
2	30	DMBA once + acetone repeatedly	acetone	–	–	–
3	30	DMBA once + Tween 60 repeatedly	acetone	11	13	6
4	30	DMBA repeatedly	acetone	9	11	4
5	20	ethanol repeatedly	ethanol	–	–	–
6	30	DMBA once + ethanol repeatedly	ethanol	–	–	–
7	20	DMBA repeatedly	ethanol	6	48	11

Discussion

These preliminary results point to an augmenting effect of ethanol on cancer formation Previous investigations on oesophagus carcinoma induction claim that

Received 21 viii 69 from the Department of Patholo[gy] [illegible]

Finland

ethanol is a syncarcinogen (*Gibel* 1967) According to the investigations presented now sole application of ethanol on animal skin does not produce tumours and thus cannot be considered as a complete carcinogen Neither does it act as a cocarcinogen in a Berenblum model experiment When used as a solvent ethanol shortens the time of latency i e the time of appearance of the first tumour and increases the tumour yield An obvious explanation is that ethanol increases the penetration of carcinogen into the tissues thus increasing the effect of the carcino enic substances In experiments with oral administration of carcinogens in ethanol (*Horie et al* 1965) many papillomas and carcinomas of the forestomach have been found but no cancer was detected among mice to which only diluted ethanol was administered In the case of man the conclusion might be drawn that ethanol does not alone pro duce tumours but perhaps it could augment the effect of small doses of carcinogens otherwise found in food and drinking fluids

References 1 *Gibel W* Arch Geschwulstf 30 181-189 1967 —2 *Gsell C & Löffler A* Dtsch Med Wschr 43 2173-2180 196. —3 *Horie A Kohschi S & Kuratsune M* Gann 56 429-441 196. —4 *Kuratsune M Kohschi S & Horie A* Gann 56 177-187 1965

Acta path microbiol scand 77 326-328 1969

BRIEF REPORT

SARCOIDOSIS

Immunohistochemical Demonstration of Immunoglobulin IgD in Sarcoid Lymph Nodes

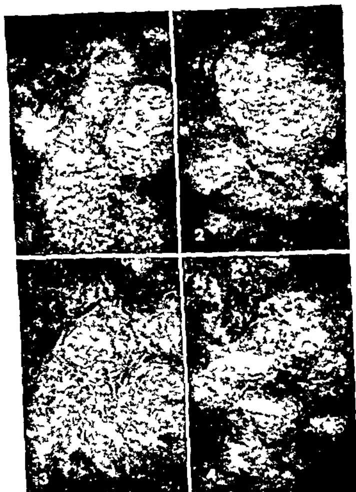
By *P Elling* and *J Wanstrup*

Rowe & Fahey 1965 described the characteristics of a new class of immunoglobulins termed IgD now shown to be present in small amounts (approximately 4 9 mg per 100 ml) in most normal sera (*Johansson et al* 1968) A specific biological or immunological function for this IgD has as yet not been demonstrated The present report is an immunohistochemical investigation which indicates that IgD is accumulated in sarcoid granulomatous tissue We have previously reported that the specific lesions in sarcoid lymph nodes contain immunoglobulin IgA IgG and IgM probably with immunoglobulin IgA as the main component (*Wanstrup & Elling* 1968)

Material and Methods

The material was obtained from 4 patients with generalized sarcoidosis Morphologically it was early active cases with cellular granulomas although hyalinosis was present in perigranulomatous localizations The tissue investigated consisted of lymph nodes from the paratracheal region After surgical removal they were frozen in isopentane and solid carbon dioxide and stored at —70 C for a few weeks until simultaneous examination could be carried out The direct immunofluorescent antibody technique was employed Antisera against the heavy chains of IgG IgA and IgM were obtained from Central Laboratory Red Cross Amsterdam

Received 1? ix 69 *from Statens Seruminstitut Copenhagen Denmark and Institute of Pathological Anatomy University of Copenhagen Denmark*



Figs 1 and 2 Sections from sarcoid lymph nodes viewed in fluorescent microscope after incubation with FITC labelled anti human IgD immunoglobulin Marked fluorescence of several granulomas × 120

Figs 3 and 4 Sections of sarcoid lymph nodes viewed in fluorescent microscope after incubation with FITC labelled anti serum against human light chains type K (Fig 3) and type L (Fig 4)

he antisera against immunoglobulin IgD were kindly supplied by *Jules J Snoijink* D Nordic Pharmaceutical Co Tilburg This IgD antisera showed monospecific actions for human IgD in normal human sera and in an IgD—myeloma serum in ilutions up to 1 64 The anti era against light chains (Kappa and Lambda) were btained from the Institute of Sera and Vaccines (Sevac) Prague They were tested y immunoelectroforesis (kindly performed by *B Mansa* M D Statens Seruminsti at) and shown to give monospecific reactions with Bence Jones proteins of Kappa nd Lambda specificity

The final F P ratio of the conjugates was in the range of 3–6 μg/g protein

The microscope was a Leitz Ortholux equipped with a Tiyoda UV superwide darkfield condensor a two band interference filter (495 nm) with a high trans mission of light and an UV blue stopping (500 nm) secondary filter (*Rygaard & Olsen* 1969)

Results and Comments

All the sarcoid lymph nodes investigated displayed an increased immunoglobulin content as compared with normal lymph nodes investigated in our previous paper Employing FITC labelled antisera against the heavy chains of immunoglobulin IgG IgA and IgM varying degrees of fluorescence were seen indicating an accumulation of these immunoglobulins in the perigranulomatous and perivascular zones with an accentuation of the reaction in most cellular granulomas This is in accordance with previous findings (*Wanstrup & Elling* 1968) Investigations with FITC labelled antisera against immunoglobulin IgD showed basically the same pattern of fluorescence In comparison with that exhibited by the antisera against IgG IgA and IgM immunoglobulins it was evident that the IgD conjugate showed a more intense reaction As illustrated in Figs 1–2 the IgD conjugate showed a strong reaction within the granulomas but did not in contrast to the other immunoglobulins seem to be present in the hyaline material The intensity of the fluorescence is generally thought to depend on the amounts FITC bound in the tissue and as the F P ratios of the different heavy chain conjugates differ only slightly our findings indicate that immunoglobulin IgD is present in sarcoid tissue in greater concentrations than other immunoglobulins

Estimations of the exact amounts of immunoglobulins or other antigens in tissue using the immunofluorescent technique are however still problematic

As apparent from Figs 3–4 the light chains of human immunoglobulins of both Kappa and Lambda specificity were found accumulated in exactly the same areas as the heavy chains Since the light chains of the immunoglobulin unit are identical in all classes these findings are only to be expected and indirectly confirm our previous and present findings i e that all of the immunoglobulins are present in the granulomatous areas although in highly varying amounts

The possibility of a common control mechanism in the synthesis of IgA and IgD has recently been postulated based on the findings of simultaneous IgA and IgD agammaglobulinaemia (*Johansson et al* 1968) Our findings of an accumulation preferentially of immunoglobulin IgA together with IgD in sarcoid tissue may support this hypothesis

References 1 *Johansson S G O Hogman C F & Killander J* Acta path microbiol scand 74 519 1968—2 *Rowe D & Fahey J* J Exp Med 121 171 1965—3 *Rygaard J & Olsen W* Acta path microbiol scand 76 146 1969—4 *Wanstrup J & Elling P* Acta path microbiol scand 73 37 1968

Acta path microbiol scand 77 329-352 1969

TRANSACTIONS OF THE SWEDISH SOCIETY FOR MICROBIOLOGY, SCANDINAVIAN SYMPOSIUM ON IMMUNOLOGY

Stockholm March 20-22 1969

Received 2 vii 69

K Penttinen G Myllylä O Makela & A Vaheri Department of Virology and Department of Serology and Bacteriology University of Helsinki and Finnish Red Cross Blood Transfusion Service Helsinki Finland SOLUBLE IMMUNE COMPLEXES AND PLATELET AGGREGATION

A micromethod involving platelet sedimentation patterns was used to determine antibodies and antigens in virus diseases Platelet aggregation (PA) appears to be caused by the reaction of soluble antigen antibody complexes with platelets The reaction was investigated with the antigenic determinant NIP coupled to BSA or HSA The molar ratio NIP BSA was 1 9 9 10 24 or 40 in different preparations Anti NIP serum produced no PA with NIP_1 BSA but all the other antigens induced PA from an Ab/Ag ratio of 0 1-0 5 upwards PA disappeared when the Ab/Ag ratio exceeded an upper limit which depended on the coupling ratio of the antigen NIP antigen produced PA at Ab/Ag = 4 and the others at Ab/Ag = 50-60 With increasing amounts of NIP per mole albumin the antibody titre increased from 1/320 to 1/1280 The smallest amount of antigen detectable was 1 6 ng/ml of NIP_{40} BSA antigen Immune complexes active in PA sedimenting at 20-25 S were demonstrated by gradient centrifugation The PA reaction was more sensitive than the complement fixation (CF) test The results support the hypothesis that the effective antigens contain at least two functional groups and that PA active soluble immune complexes are formed also in antibody excess

C G Gahmberg & K Simons Department of Serology and Bacteriology University of Helsinki Finland ISOLATION OF PLASMA MEMBRANE FRAGMENTS FROM BHK 21 CELLS

A fraction rich in plasma membrane was isolated from baby hamster kidney cells (BHK 21) by a modification of the method of *Bosmann et al* (Arch Biochem Biophys 128 51 1968) used for Hela cells

The specific activities of 5 nucleotidase Na+ - K+ - activated ATPase and acid phosphatase were 8-10 times higher in the membrane fraction than in the homogenate whereas NADH diaphorase and succinate dehydrogenase activities were lower than in the *homogenate Electron* microscopy showed that the membrane preparations were not contaminated by other identifiable subcellular components The membranes were solubilized in 1 per cent SDS and 1 per cent 2 mercaptoethanol and run in a SDS polyacrylamide disc electrophoresis at a basic pH A complex banding pattern was obtained after staining with Coomassie blue

Antiserum was made against whole BHK cells in rabbits Cells were incubated with this antiserum and fluorescein labelled sheep anti-gamma globulin serum was

added The plasma membranes of the BHK cells showed a strong fluorescence If the antiserum was absorbed with isolated membranes no membrane fluorescence was obtained

Fred Blomberg Wenner Gren Institute Stockholm Sweden IMMUNOCHEMICAL STUDIES OF PLASMA MEMBRANES FROM RAT LIVER CELLS

Plasma membranes were isolated by standard procedures The purity of the preparation was checked by phase contrast microscopy and assay of membrane associated enzyme activities Antisera against these membrane were prepared in rabbits and compared with similar antisera prepared against different intracellular rat liver membranes belonging to the endoplasmic reticulum of the parenchymal cells The different types of membranes *were characterized by a considerable number* of antigens specific for each of them Solubilization of plasma membranes by different methods and combination of the agar diffusion test with histochemical enzyme staining of the resulting precipitates led to the recognition of at least 10 different components not found in any of the other subcellular fractions studied. *Some of these antigens were characterized as enzymes Fluorescent antibody staining* of liver slices with the anti plasma membrane serum provided good additional evidence of the membrane specificity of the antibodies These stained liver plasma membranes in general and were not specifically directed against antigens of reticulo endothelial origin

F Celada Department of Tumor Biology Karolinska Institute Stockholm Sweden *ACTIVATION OF A DEFECTIVE E COLI β GALACTOSIDASE BY ANTIBODIES* CHARACTERISTICS OF THE ANTIGENIC DETERMINANT INVOLVED

A striking example of activation of enzymes by antibody was recently found (1) in vitro incubation with serum from rabbits or mice immunized against *F coli* B galactosidase caused a 1 000 fold increase in the hydrolising potency of the defective gene product of a point *lac* mutant (a) While the antigen antibody reaction is extremely rapid the conformational change that it induces in the mutant molecule leading to the recovery of enzyme activity is a relatively slow process (b) The interaction of monovalent antibody fragments with a specific site of the mutant enzyme is sufficient to bring about activation The dose effect relationship is compatible with a one hit kinetics (c) Like all other antigenic determinants the specific site on which activation depends is common to the wild type and the mutant enzyme molecule however it is not immunogenic in the latter (d) *Tolerance to the* activating site is induced by injection of the mutant enzyme in newborns

T Tallberg Department of Serology and Bacteriology University of Helsinki Finland DETECTION OF ANTIBODY OR ANTIGEN BY SLIDE AGGLUTINATION TESTS USING IMMUNOLOGICALLY COATED ANTIBODY ACTIVE PROTEIN POLYMER PARTICLES

The preparation and use of small water insoluble antigen or antibody active polymer particles as indicators in a slide agglutination method for strong *immune* reactions *e g* albumin anti albumin has been described *previously (Tallberg Ann* Med exp Fenn 45 477 1967)

Weak antigens like the protein hormone Human Placental Lactogen (HPL) did not cause agglutination when added to anti HPL active particles

The antigenicity of HPL was lost during polymerization Anti HPL particles in cubated with HPL at equivalence or slight antigen excess became immunologically coated After three days at +4 C the contaminants were removed by centrifugation in PBS and the particles were resuspended and homogenized by ultrasound After sedimentation by gravity or light centrifugation a drop of the opalescent super natant was agglutinated on glass plates in two minutes by adding a drop of anti HPL serum in dilutions 1 40–1 1600 Agglutination could be inhibited by pure HPL or a drop of serum from pregnant women

Hans Erik Carlsson Wenner Gren Institute Dept of Immunology Norrtullsgatan 16 S 113 45 Stockholm Sweden ISO FLECTRIC FOCUSING AND IMMUNOLOGI CAL CHARACTERIZATION OF HUMAN SERUM ALBUMIN

A purified preparation of HSA was subjected to electrofocusing in carrier am pholyte at the pH range 4-6 The protein content of the eluate was determined spectrophotometrically at 280 nm and the fractions were also analysed immuno chemically by immunoelectrophoresis and by qualitative and quantitative agar diffusion

Electrofocusing resulted in the appearance of three HSA containing fractions with isoelectric points of pH ~ 4 6 ~ 4 8 and ~ 5 1

The pH 5 1 fraction contained appr 60 per cent of the HSA recovered No im munochemical differences were found between HSA in the different fractions

M Cesla F Grossmüller & U Lundkvist Department of Biochemistry Pharmacia AB Uppsala Sweden RADIO IMMUNO ASSAY OF INSULIN BY MEANS OF ANTIBODY COATED TEST TUBE

Antibodies to insulin were adsorbed to the inner walls of disposable polystyrene tubes These tubes were used for solid phase radio immuno assay of insulin The ound antigen was separated from the free antigen by washing the tubes with buf er solutions or distilled water

Linder & T Tallberg Department of Serology and Bacteriology University of Hel sinki Helsinki Finland PREPARATION OF FLUORESCENT ANTI IMMUNO GLOBULIN SERA WITH HIGH SPECIFIC ACTIVITY USING IMMUNO AD SORBENTS

The highly specific antisera required for the indirect immunofluorescence techni que are difficult to produce We used immuno adsorbents (*Avrameas & Ternynck* J Biol Chem 242 1651 1967) to produce immunologically specific anti immuno globulin sera and avoid labelling other than the specific anti immunoglobulin anti bodies with fluorescein iso thiocyanate (FITC)

Immunoglobulins from human sera with anti tetanus toxoid titres 10 7 were adsorbed on tetanus toxoid antigen polymers The immunoglobulins were then eluted with NaI 100 µg of this in complete Freund s adju ant were used to im munize sheep

Polymerized human serum was then used to adsorb the specific immunoglobulins from these sheep antisera Anti IgG and anti IgM were eluted from the adsorben

Sheep antibodies were conjugated with FITC isomer I The fluorescein was a lowed to diffuse gradually through a dialysis bag into the surrounding protei

added The plasma membranes of the BHK cells showed a strong fluorescence If the antiserum was absorbed with isolated membranes no membrane fluorescence was obtained

Fred Blomberg Wenner Gren Institute Stockholm Sweden IMMUNOCHEMICAL STUDIES OF PLASMA MEMBRANES FROM RAT LIVER CELLS

Plasma membranes were isolated by standard procedures The purity of the preparation was checked by phase contrast microscopy and assay of membrane associated enzyme activities Antisera against these membranes were prepared in rabbits and compared with similar antisera prepared against different intracellular rat liver membranes belonging to the endoplasmic reticulum of the parenchymal cells The different types of membranes were characterized by a considerable number of antigens specific for each of them Solubilization of plasma membranes by different methods and combination of the agar diffusion test with histochemical enzyme staining of the resulting precipitates led to the recognition of at least 10 different components not found in any of the other subcellular fractions studied. Some of these antigens were characterized as enzymes Fluorescent antibody staining of liver slices with the anti plasma membrane serum provided good additional evidence of the membrane specificity of the antibodies These stained liver plasma membranes in general and were not specifically directed against antigens of reticulo endothelial origin

F Celada Department of Tumor Biology Karolinska Institute Stockholm Sweden *ACTIVATION OF A DEFECTIVE E COLI β GALACTOSIDASE BY ANTIBODIES* CHARACTERISTICS OF THE ANTIGENIC DETERMINANT INVOLVED

A striking example of activation of enzymes by antibody was recently found (1) in vitro incubation with serum from rabbits or mice immunized against *F coli* B galactosidase caused a 1 000 fold increase in the hydrolising potency of the defective gene product of a point *lac* mutant (a) While the antigen antibody reaction is extremely rapid the conformational change that it induces in the mutant molecule leading to the recovery of enzyme activity is a relatively slow process (b) The interaction of monovalent antibody fragments with a specific site of the mutant enzyme is sufficient to bring about activation The dose effect relationship is compatible with a one hit kinetics (c) Like all other antigenic determinants the specific site on which activation depends is common to the wild type and the mutant enzyme molecule however it is not immunogenic in *the latter* (d) *Tolerance* to the activating site is induced by injection of the mutant enzyme in newborns

T Tallberg Department of Serology and Bacteriology University of Helsinki Finland DETECTION OF ANTIBODY OR ANTIGEN BY SLIDE AGGLUTINATION TESTS USING IMMUNOLOGICALLY COATED ANTIBODY ACTIVE *PROTEIN* POLYMER PARTICLES

The preparation and use of small water insoluble antigen or antibody active polymer particles as indicators in a slide agglutination method for strong immune reactions *e g* albumin anti albumin *has been described previously* (*Tallberg* Ann Med exp Fenn 45 477 1967)

Weak antigens like the protein hormone "Human Placental Lactogen" (*HPL*) did not cause agglutination when added to anti HPL active particles

H Bennich S G O Johansson & D R Stanworth Blood Center University Hospital Uppsala Sweden and Department of Experimental Pathology University of Birmingham Birmingham England EFFECT OF REDUCTION UPON THE BIOLOGICAL AND IMMUNOLOGICAL PROPERTIES OF IMMUNOGLOBULIN E

Immunoglobulin E contains 21 disulphide bonds per 200 000 molecular weight 9-10 of which are susceptible to reduction with mercaptans in aqueous media at pH 8 The single bond which links a light polypeptide chain to the epsilon chain is attacked as rapidly as the corresponding bond linking a light chain to a γ polypeptide chain

Since the remaining 7 to 8 disulphide bonds which react relatively slowly were found to represent inter and intra chain bonds characteristic of the epsilon polypeptide chains their relation to the antigenic and the skin fixing properties of IgE were studied further

Quantitative antigenic analysis showed that the cleavage of 2 5 bonds did not significantly alter the antigenicity of the molecule whereas cleavage of bonds preferentially located within the C terminal portion of the epsilon chains drastically changed the antigenic characteristics of IgE Isolated and partially reduced epsilon chains give weak antigenic reactions but retain their immunogenic properties whereas completely reduced epsilon chains have apparently lost antigenic as well as immunogenic properties

IgE specifically inhibits the Prausnitz Kustners (P K) reaction in healthy individuals and the passive cutaneous anaphylactic (PCA) reaction in monkeys The results from inhibition experiments of the PCA reaction with reduced IgE indicate that the blocking i e the skin fixing properties are lost when more than 5 of the 9 susceptible bonds are cleaved

The similar effect of reduction upon structures responsible for specific antigenic determinants and skin fixing properties respectively taken together with the finding that both kinds of activities can be recovered in the Fc fragment indicates that they are closely related and occupy a very limited portion of the epsilon polypeptide chains or alternatively occupy regions in close proximity

J B Natvig Research Inst of Rheumatology Rikshospitalet and Oslo Sanitets forening University Hospitals Oslo 1 Norway ANTIGENIC MARKERS OF IMMUNOGLOBULIN HEAVY CHAINS

Immunoglobulin heavy chains can be studied using various antigenic structures detected by specific antisera There are several types of antigens First the isotypes which are antigens present in all normal human sera They specify the five immunoglobulin classes and furthermore four G and two A subclasses However there are also isotype antigens shared by some but not all G subclasses thus reflecting some of the common sequences which have been preserved during evolution of the subclasses (1) Secondly the allotypes such as the Gm markers in man are antigens present in some but not all normal sera

First the appearance of Gm allotypes may result from genetic events in part of the gene specifying one particular subclass the two independent gene sequences behaving as true alleles Gmf and Gmz may represent true alleles specified by homologous base sequences in the gene

Secondly a genetic event may occur in a part of the gene common ... one subclass This may give rise to one structure specifying an ordi... type However the antitetic marker corresponds to a structure which

in other subclasses and the respective antigens show a very distinct distribution. They behave like allotypic markers within one subclass However since such an antigen is present in all proteins of one or two other subclasses it behaves like an isotype in whole human sera Two such markers non a and non g have been described (1) Non a was present in the Gm(a—) Fc fragments of the G1 subclass and behaved as being determined by the allele of *Gma* It was also present in all G2 and G3 proteins Non g was present in the Gm(g—) Fc fragments of the G3 subclass and behaved as being determined by the allele of *Gmg* However all G2 proteins were non g positive

P Brandtzaeg Institute of Pathological Anatomy Rikshospitalet Oslo A NEW ANTIGENIC DETERMINANT OF SECRETORY IMMUNOGLOBULIN A

Immunoglobulin A (IgA) of human external secretions is associated with a protein called secretory piece (SP) which is absent from normal serum

Rabbits immunized with normally occurring free SP from parotid secretion produced antibodies to an antigenic determinant not detectable on SP associated with IgA The antigenic group was shown to be hidden in the secretory IgA molecule By reduction and alkylation of the latter the group was made accessible on molecules of SP released these exhibited similar antigenic and size characteristics as normally occurring free SP By treating secretory IgA with 6M urea at pH 3 5 the hidden antigenic group was made accessible without concurrent splitting of the composite molecule

By means of fluorescent antibodies specific for the new antigenic determinant it was shown that SP molecules occur free in acinar epithelial cells Neither free nor bound SP could be detected in nearby plasma cells and connective tissue ground substance which however contained IgA

M Harboe B C Solheim & J Deverill Institute for Experimental Medical Research Ullevål Hospital Oslo Norway SPLITTING AND REASSOCIATION OF MACROGLOBULINS

Reducing agents split the gamma M molecule into its five subunits

The localization of various idiotypic antigenic determinants on the intact gamma M molecule the subunit and reassociated protein was studied Some idiotypic antigenic determinants were present both on the intact gamma M molecule and on the subunit the latter was often unable to precipitate with the corresponding antibody but combined with it and inhibited precipitation Other determinants were apparently destroyed by mild reduction of the gamma M molecule but were re formed by reassociation

Reduction and reassociation of mixtures of two monoclonal gamma M globulins resulted in formation of hybrid molecules containing subunits of different origin Antisera reacting with idiotypic antigenic determinants were useful for a demonstration of hybrid formation Hybrid molecules retained antibody activity

T Eskeland & M Harboe Anatomical Institute and Institute for Experimental Medical Research University of Oslo Norway ELECTRON MICROSCOPIC STUDIES OF NATIVE AND REASSOCIATED γM

Using the electron microscope and negative contrast technique *Chesebro et al* (1) have studied purified M

Our native material consisted of one purified human monoclonal γM In the electron microscope using the negative contrast technique one or several arms were split at the tip thus resembling a Y which indicates that all the five arms of a γM molecule can exhibit this structural feature Since the antibody-combining sites are located near the tip of the arms the observation implies that the γM molecule has ten potential combining sites

Reassociated fast sedimenting protein was isolated by sucrose density gradient ultracentrifugation Electron microscopy clearly showed that some of the reasso ciated molecules consisted of five arms or subunits and some of four This may explain why reassociated material sediments a little slower than native material

S E Svehag & B Bloth Department of Immunology National Bacteriological La boratory and Department of Immunology Karolinska Institute Stockholm
ULTRASTRUCTURE AND BIOLOGICAL ACTIVITY OF SUBUNITS AND PRO TEOLYTIC FRAGMENTS OF IgM GLOBULINS

The IgM molecule has the form of a flexible circular pentameter Subsequent studies have sought to relate the ultrastructure of IgM subunits and of the papain and pepsin digestion fragments of IgM to the fine architecture of the intact IgM molecule The subunits (90 × 35 A) were found to represent the filamentous ap pendages extending from the central ring structure in the intact molecule They retained only 7 to 12 per cent of the antibody activity of the intact molecule bound weakly to antigen and the majority of the subunits were univalent Reaggregated IgM antibody retained about 50 per cent of the initial antibody activity

Papain digested Fc fragments were found to make up the central ring structure (outer diameter 85 A) in the intact IgM molecule plus a minor part of the appen dages extending from the ring The Fcμ fragments were occasionally seen to be composed of dimers of short rods probably identical with two endpieces of μ-chains These findings provide morphological evidence that the Fc fragments containing the C terminal ends of the μ chains make up the central ring structure in the IgM molecule The Fabμ and F(ab) μ fragments were located on the appendages ex tending from this ring The F(ab) μ fragments constituted about 3/4 of the ap pendages

P Toivanen & T Hirvonen Department of Medical Microbiology and Department of Obstetrics and Gynecology University of Turku Turku 3 Finland
ISO AND HETERO AGGLUTININS IN HUMAN FOETAL AND NEONATAL SERA

AB and sheep cell haemagglutinins were studied with saline technique in cord sera of human foetuses from different stages of gestation and of full term newborns At the earliest they were found in foetuses after the 13th to 17th week of gestation In those aged 18 weeks or more they were observed as frequently as in full term newborns 57 per cent of which had iso agglutinins and 58 per cent hetero agglu tinins The maternal origin of iso agglutinins in the foetal sera is indicated by their coinciding with the same iso agglutinin in the maternal serum by their independence on the ABO group of the foetus and by the fact that iso agglutinins in neonatal sera generally belong to the IgG class The maternal origin of sheep cell haemagglutinins is suggested by their IgG nature in neonatal sera

Iso agglutinins of foetal origin that is active against erythrocytes of the mother s group were detected in eight out of 44 neonatal sera displaying a potential for those but in none of ten sera from foetuses aged 14 to 19 weeks

K Nilsson J Ponten L Philipson & J Sjökvist Wallenberg Laboratory University of Uppsala, Uppsala Sweden MONOCLONAL IMMUNOGLOBULIN PRODUCTION IN VITRO BY ESTABLISHED HUMAN LYMPHOID CELL LINES

Permanent lymphoid cell lines have successfully been established in long term cultures from normal and malignant human lymphoid tissue with a modified Trowell-Jensen grid procedure Morphologically the established cell lines consist of blastoid cells in the lymphoid series The cells preserve a highly differentiated function reflected by production of IgG IgA or IgM

Immunoglobulins have been assayed by incubation of lymphoid cells with a 14C amino acid mixture So far 12 cell lines have been found to produce IgG 2 lines IgA and 3 lines IgM The light chain has mainly been of kappa type One line produced only kappa light chains

To establish the monoclonal nature of synthesized IgG normal human IgG was added to the concentrated supernatants *from two separate cell line cultures The* result is consistent with the fact that ligh chains from a monoclonal IgG (myeloma proein) in starch gel electrophoresis at pH 7–8 produces only one or two bands *In addition the experiments show* that *intact IgG* was *synthesized by the cells* since isolated heavy and light chains were both radioactivel labelled

J Tamvik Gades Institute Bergen Norway SECONDARY IMMUNE RESPONSE IN RABBIT BLOOD LYMPHOCYTES STIMULATED IN VITRO WITH SHEEP RED CELLS

Blastoid transformation plaque forming cells and agglutinins against sheep red cells (SRC) developed in SRC stimulated cultures containing blood lymphocytes from SRC immunized rabbits *The* majority *of the lytic* plaques were small microscopically visible in SRC monolayers No definite liberation of lytic antibodies into the culture media was detected. However agglutinins against SRC were regularly found during the second week of culturing concomitant with the appearance of plasma cells in the cultures The agglutinins showed the physico-chemical characteristics of IgG

B Andersson & H Wigzell Department of Tumor Biology Karolinska Institutet, *Stockholm* Sweden STUDIES ON THE AVIDITY OF ANTIBODIES PRODUCED BY SINGLE CELLS

Mice were immunized with a high or a low dose of BSA (5000 μg or 50 μg) The inhibition curves obtained *by adding free antigen to the agar plates in the haemo*lytic plaque assay for single antibody producing cells were also studied. *There was* a parallel change in the avidity at the serum antibody level and in the inhibition characteristics at the cellular level during *the first three weeks after immunized* with a high dose of antigen showed a lower avidity than those receiving a low dose

Cell populations producing antibodies of high and *low avidity respectively* were mixed and thereafter allowed to pass through a column *of plastic beads* coated with the antigen or normal mouse serum proteins The *average avidity* of the antibodies was determined at the cellular level before and after column passage The cells passing the antigen coated column were producing *antibodies* of lower avidity than the cells which were retained in the column *The cell bound* receptor and *the produced* antibodies show similar binding *characteristics* for the antigen

J Holmgren & L A Hanson Institute of Medical Microbiology Department of Bacteriology University of Goteborg Goteborg Sweden THE ANTIBODY RESPONSE IN RABBITS TO AN E COLI K ANTIGEN

Some evidence has been presented indicating that the capsule of E coli bacteria is an important virulence factor and antibodies to K antigens are protective against infection

The K antigen in *E coli* bacteria of the serotype 06 K?a ?c H1 was isolated The K antibody response in rabbits to a single and to a booster intravenous injection of 10^8 formalin killed *E coli* 06 K?a 2c H1 bacteria was studied It was found that after the single injection into rabbits a rapid agglutinin response was seen A rapid decline was then observed (average 50 per cent reduction each 5½ day) suggesting that an almost complete interruption of the synthesis of antibodies was mainly of IgM type After the booster injection the passive haemagglutination titres rose in all rabbits to a level higher by two titre steps i e fourfold than the highest titres measured after the first injection As after the first injection very little of reduction resistant antibodies were seen

S Britton Department of Bacteriology Karolinska Institutet Stockholm Sweden INDUCTION OF PARALYSIS BY ANTIGEN EXPOSURE OF LYMPHOID CELLS IN VITRO

Normal mouse lymphoid cells have been exposed to various doses of soluble antigen in vitro and under varying conditions Under certain conditions it was possible to induce paralysis as judged by the inability of such treated cells to respond to an immunizing dose of antigen in a secondary irradiated host The conditions favouring induction of paralysis will be described and the question whether the induction takes place in vitro or in the later in vivo milieu will be discussed

A Sakai N Müller Berat & J O Vang Department of Experimental Surgery Lund Sweden THE EFFECT OF PARTIAL HEPATECTOMY ON ANTIBODY RESPONSE

The central role of the liver in protein metabolism and the fact that major resection of the liver not only leads to increased DNA synthesis in the regenerating liver cells but also in lymphoid tissues as measured by the uptake of radiolabelled thymidine indicates that the liver may play a more general role in modifying the antibody response to any given antigen

A series of experiments have been carried out to explore the effect of partial hepatectomy on the antibody production in the rat following immunization with sheep blood red cells

SRBC were injected intraperitoneally into Wistar rats At varying intervals after the injection a 70 per cent liver resection was performed

The results show that partial hepatectomy causes a rise in reacting antibody equal to or in excess of that noted in the control groups in which the hepatectomy was substituted with a second challenge of SRBC

A rise in haemagglutinin titre was noted already 10 20 hours after liver resection This initial rise was however only transient but it was followed by a steady increase in titre the following days

J H Larsen Institute of Medical Microbiology University of Copenhagen Copenhagen Denmark THE CONTENT OF A SOLUBLE ANTIGEN IN THE ORGANS OF TOLERANT MICE AND MICE INFECTED INTRAPERITONEALLY WITH LCM VIRUS

The well known LCM virus carrier state of mice has previously been shown to be a state of immunological tolerance The mechanism of this tolerance is unknown but the author has put forward the hypothesis that this tolerance is a state of specific immunosuppression possibly caused by the permanent presence and multiplication of the virus in the specifically reactive cells

The kidneys spleens livers and brains have been investigated for the contents of infective virus (LD_{50}) and of a soluble specific antigen (CF) The following categories of animals were investigated tolerant virus carrier mice normal mice and intraperitoneally infected mice In the case of the intraperitoneally infected mice the virus titres were found to be increased during the first week after the inoculation Maximum titres were obtained during the second week The content of soluble antigen also increased analogously

In the organs of the tolerant virus carriers large amounts of antigen were found together with high virus titres It was found that although the virus titres of the tolerant virus carriers and the maximum titres of the intraperitoneally infected mice were at the same level the content of soluble antigen was much larger in the organs of the virus carriers than in the organs of the intraperitoneally infected mice

L Lindholm L A Nilsson L Rydberg I Andersson C S Britton Department of Bacteriology University of Goteborg Goteborg Sweden INTERCELLULAR TRANSFER OF DNA A POSSIBLE MEANS OF COMMUNICATION BETWEEN IMMUNOCYTES

It has been demonstrated by *Millet et al* that following stimulation of mice with sheep erythrocytes thymus derived lymphocytes react with the antigen and subsequently interact with marrow derived lymphocytes which develop into antibody forming cells

Staining of mono layer cultures of thymus and bone marrow cells with the Feulgen stain revealed the presence of bridges containing DNA between individual lymphocytes Electron microscopic studies of such bridges in ultra thin sections confirmed the presence of DNA within bridges between thymus and bone marrow cells Furthermore time lapse cinematography of mixed cultures of thymus and bone marrow cells revealed frequent formation of connections between the cells The ability of thymus and bone marrow cells to establish contact between one another has been measured in experiments where thymus or marrow cells were agglutinated onto mono layers of HeLa cells and ^{51}Cr labelled syngeneic thymus or bone marrow cells subsequently added Following incubation the amount of labelled cells adhering to the bottom layer of thymus or marrow could be measured These studies indicate that marrow cells bind preferentially to syngeneic thymus cells

Electron microscopy of ultra thin sections from the thymus has given evidence that DNA may be transferred also between cells in close contact within the intact thymus

J Andersson D Killander E Möller & *G Moller* Institute for Medical Cell Research and Genetics and Department of Bacteriology Karolinska Institutet Stockholm Sweden RAPID LYMPHOCYTE RECOGNITION OF HISTO INCOMPATIBILITY CHANGES OF THE DEOXYRIBONUCLEOPROTEIN COMPLEX IN MIXED CULTURES OF ALLOGENEIC LYMPHOCYTES

The mechanism of growth stimulation in mixed lymphocyte cultures was studied at the individual cell level using cytophotometric techniques Human lymphocytes were fixed and stained with acridine orange (AO) By means of microspectro fluorimetry a pronounced increase in fluorescence intensity of AO stained lym pho ytes was observed as early as 1–3 hours after stimulation with foreign trans plantation antigens in mixed lymphocyte cultures

This increase in fluorescence intensity was not due to an increased amount of DNA but to an increased accessibility of AO binding sites in the deoxyribonucleo protein (DNP) complex The reaction occurred in all cells only when the donors of the lymphocytes were unrelated and incompatible at the major histocompatibility locus (HL A) In mixed cultures where donors were compatible at this locus no increase in Ao binding to DNP was observed

The AO binding reaction probably reflects a specific recognition of HL A anti gens whereas other antigenic discrepancies between the individuals do not cause an analogous response

N R Ringert Z Darzynkiewicz & *L Bolund* Institute for Medical Cell Research and Genetics Karolinska Institutet Stockholm Sweden CHROMATIN CHANGES IN PHA STIMULATED HUMAN LYMPHOCYTES

Stimulation of lymphocytes by PHA induces marked changes in the cytochemical properties of nuclear chromatin These changes manifest themselves in an in creased acridine orange (AO) binding to deoxyribonucleoprotein (DNP) an in reasing sensitivity of DNP to heat denaturation and altered staining properties of the histone component of DNP

It was demonstrated that only a fraction of the lymphocyte population respond to PHA stimulation with an increased rate of RNA synthesis The same proportion of cells shows an increased capacity to bind ^{3}H actinomycin D (^{3}H AMD)

It has previously been suggested that gene activation in PHA stimulated lympho tes involves acetylation of histone amino groups Double emulsion autoradio graphy after labelling of PHA stimulated cells with ^{14}C acetate and ^{3}H uridine sug gests that they are the cell which acetylate histone which start to synthesize RNA

Taken together these results suggest that gene activation in PHA simulated lym phocytes involves several steps One of the first steps referred to as chromatin ac tivation is reflected in the increased AO binding increased sensitivity to heat denaturation and altered histone staining of the DNP

H Wigzell & *B Andersson* Department of Tumor Biology Karolinska Institutet Stockholm Sweden SEPARATION OF ANTIGEN SENSITIVE CELLS BY ANTI GEN COATED BEAD COLUMNS

When antigen sensitive cells are allowed to pass through antigen coated columns he cells specifically reactive against the antigen used for coating the column are tained within the column Cells reactive against other antigenic specificities are not eliminated from the passing cell population This specific separation could be

shown to take place whether the cells were derived from immune or normal animals

Blocking of specific elimination by the antigen coated columns could be obtained by using free antigen in the column medium durin separation

The results obtained are considered to be in strong support of a cell associated antibody on the outer surface of antigen sensitive cells Results will be presented suggesting the active production of the cell associated receptor by the antigen sensitive cells

Cells reactive against haptenic groups could be separated from those reactive against carrier specific antigens

S K Singhal & H Wigzell Department of Tumor Biology Karolinska Institutet Stockholm Sweden COGNITION OF ANTIGEN BY NORMAL BONE MARROW CELLS

DNA synthesis of normal bone marrow cells can be increased by the addition of a number of protein antigens in vitro The antigen sensitive cells of the bone marrow have the appearance of the small lymphocyte We have recently studied the immunological reactivity of bone marrow cells utilizing antigen coated bead columns through which normal bone marrow cells are allowed to pass When stimulated by the same antigen as that used in the column the in vitro DNA synthesis was found to be much greater in the retained and mechanically eluted cells than in the passing cell populations The results strongly suggests that the antigen sensitive cell of the normal bone marrow is expressing its immunological capacity and potential reactivity at the outer cell surface

J G Iversen Institute of Physiology University of Oslo Oslo Norway ANTISERA AGAINST RECIRCULATING AND NON RECIRCULATING LYMPHOCYTES IN THE RAT

There is a large degree of lymphocyte recirculation between blood and lymphoid tissue By thoracic duct drainage for 3 days it is possible to deplete rats of recirculating lymphocytes (RL) leaving only non recirculating lymphocytes (NRL) in the blood of the animal It has been demonstrated that these two groups behave differently in vitro since only RL and not NRL are stimulated by phytohaemagglutinin to blastoid formation

The aim of the present study was to investigate whether these two lymphocyte groups also differed in their antigenic properties

Antibodies specific for RL or NRL could be demonstrated with the aid of absorbtion techniques

The results indicate that only RL are present in the thymus whereas about 20 per cent of the blood lymphocytes and most of the bone marrow lymphocytes belong to the NRL group

M Virolainen & A Lahti III Department of Pathology University of Helsinki Finland IDENTIFICATION OF MACROPHAGES WITH IMMUNOFLUORESCENT STAINING

Anti macrophage serum (AMS) was obtained from rabbits immunized with mouse peritoneal macrophages which had been cultured in vitro for two weeks AMS was cytotoxic to mouse peritoneal macrophages In addition unabsorbed AMS was cyto-

toxic to lymphocytes and erythrocytes Absorption with lymph node cells and erythrocytes indicated that these effects were due to distinct antibodies rather than to a cross reaction In double diffusion precipitation AMS gave a clear precipitation line with homogenates of cultured macrophages and fresh peritoneal exudate cells but not with bone marrow lymph node or thymus cell homogenates

Indirect immunofluorescent staining was performed with FITC conjugated anti rabbit Ig prepared in goat Acetone fixed peritoneal macrophages cultured in vitro showed a strong fluore cence in the cytoplasmic membrane In many cells fluor escence seemed to be located in the nuclear membrane too whereas the cytoplasm and the nucleus were negative Membrane fluorescence was also observed in cultured mouse alveolar macrophages and in rat peritoneal macrophages In mouse bone marrow cultures the number of cells showing membrane fluores ence paralleled the appearance of macrophages as revealed by Giemsa staining Erythroid and myeloid cells and cultured fibroblasts were negative

The results indicate that macrophage specific antigen(s) is located in the cell membrane and is not species specific

T U Kosunen Department of Serology and Bacteriology University of Helsinki Helsinki Finland ORIGIN OF MONONUCLEAR CELLS IN DELAYED HYPER SENSITIVITY REACTIONS

Two groups of inbred rats were immunized the donors received injections of 45 Lf of diphtheria toxoid (DT) in complete Freund s adjuvant the re ipients receiving DT and bovine serum albumin (BSA 0 5 mg) in adjuvant Twelve days later sepa rate cell suspensions were made from various donor organs The cells were labelled in vitro by incubation for one hour at 37 C with triti ted leucine The recipients were skintested and 4 hours later re eived one of the cell suspensions intravenously Biopsies of the skin test sites were tal en 20 hours later for radio auto raphy The number of labelled cells in 100 mi roscope fields was counted and corrected to corr spond to the actual number of labelled cells from the draining lymph nodes Comparable BSA DT and tuberculin reactions contained 89–97 labelled cells from the draining lymph nodes 29–40 from the axillary lymph nodes 16 25 from the spleen less than one from the thymus and 144–189 from the bone marrow In non immune turpentine reactions 129 labelled bone marrow cells 17 spleen cells and about one cell from each of the other organs were found In the recipient sple n 43 labelled bone marrow cells and 193–280 cells from the other organs were found Thus a large proportion of the cells in delayed skin reactions and non specifi inflammatory lesions were derived from the bone marrow

K E Fichtelius C Sundstrom B Kullgren & J Finna Institute of Histology Upp sala University Uppsala Sweden THE LYMPHO EPITHELIAL ORGANS OF HOMO SAPIENS REVISITED

Both thymus and bursa Fabri ii are defined as central lymphoid organs responsible for the development of the peripheral lymphoid tissues They are both lympho epithelial in character All animals phylogenetically distal to the lamprey exert a bursa dependent function immunoglobulin production but only the birds have a bursa Fabricii

Existing lympho epithelial relationships in bursaless vertebrates have been dis cussed A diffuse bursa may still be functioning in many animals as their only bursa and in other animals as one part of a morphologically and functionally

divided bursa organ Such a hypothetical bursa could be the gut epithelium with its lymphocytes and some *of the lymphocytes within* the lamina propria

This new look on the lympho epithelial organs makes it possible for us to discover new organs in the human body where we did not see them before Pictures will be shown of lympho epithelial micro organs of newborn children sites of exposure are the skin the bronchial tree oesophagus and the salivary glands including pancreas

A theory about the very early phylogeny of immunity will be presented According to this theory the formation of lympho epithelial organ have been induced by the presence of antogens at the outer and inner surfaces of the primitive organism

P Biberfeld C Holm & P Perlmann Patologiska avd Sabbatsbergs sjukhus och immunologiska avd Wenner Grens institut CYTOTOXIC LYMPHOCYTE TARGET CELL INTERRELATION IN VITRO

In the presence of phytohaemagglutinin (PHA) monolayers of Chang cells were destroyed by highly purified suspensions of lymph cytes from human peripheral blood The damage of the Chang cells was confined to the area covered by lymphocytes Light and electron microscopy revealed that lymphocytes established contact *with the monolayer cells* by *broad processes* and became attached by *spreading* out on the surface of the target cells Attached lymphocytes displayed signs of intense peripolesis like motility over the surface of target cells 24 to 48 hours after the addition of lymphocytes and PHA most Chang cells in the culture medium were found to be dead or irreversibly damaged Target cells still attached to the substratum did not appear to be damaged even when covered with many lymphocytes

Addition of heat inactivated anti lymphocyte serum to the culture medium inhibited the lymphocyte induced damage of the target cells The lymphocytes were agglutinated to each other and peripolesis was rare Electron microscopy of ALS treated lymphocytes showed a fuzzy coating of 100 to 200 A on the surface of these cells

G Holm & P Perlmann the Wenner Gren Institute Stockholm CYTOTOXIC ACTION OF UNSENSITIZED *LYMPHOID* CELLS *AGAINST ANTIBODY COATED* TISSUE CULTURE CELLS

As a model of cell damage in vitro erythrocytes coated with PPD were lysed by non sensitized lymphocytes in the presence of antibodies to PPD

In a similar model Chang cells labelled with ^{51}Cr chromate were treated for 30 min with heat inactivated antiserum from rabbits hyper immunized with *Chang* cells After washing 10^5 Chang cells were incubated for 1–24 hr with leucocyte containing 80–95 per cent small lymphocytes Cell damage was calculated *as the* release of radioactivity into the medium In the presence of complement but no leucocytes the target cells were not damaged by *antiserum dilutions above 1 100* In contrast leucocytes damaged target cells treated with antiserum *diluted 10^8 10* times Addition of complement was not needed Cell damage was preceded by aggregation of lymphocytes in the antibody coated target cells *Rabbit anti human* lymphocytic serum prevented aggregation and cell damage

Kinetic experiments revealed that cell damage is optimal *after 3 6 hr of incula*tion There is a semilogarithmic relation between lysis and leucocyte target cell ratio

R E Falk Judith Falk & G Möller Laboratory for Transplantation Immunology Serafimerhospital and Department of Bacteriology Karolinska Institutet Stockholm Sweden EFFECT ON HOMOGRAFT SURVIVAL OF ANTISERA PREPARED AGAINST SUPERNATANTS FROM CULTURES OF SENSITIZED LYMPHOCYTES MIXED WITH THE CORRESPONDING HISTOCOMPATIBILITY ANTIGENS

It has been demonstrated that rodent spleen cells or human blood leucocytes from donors sensitized to transplantation antigens can be inhibited in their migration from capillary tubes by the specific sensitizing antigen The mechanism of the inhibition is similar to that demonstrated in delayed hypersensitivity states the release by sensitized lymphocytes of active soluble substances capable of producing similar activation of normal lymphocytes

Antisera were produced by immunizing rabbits with active supernatants from rat lymphocytes mixed with equal parts of Freund's complete adjuvant Three weeks later the rabbits were given weekly intramuscular injections of similar supernatants for 2 to 6 weeks One week after the last injection they were exsanguinated the sera separated and the globulin precipitated with ammonium sulphate Rat were injected with from 1 to 3 mg/g body weight of the globulin fraction prior to and after receiving an allogeneic skin graft Four different lots of gamma globulin were tested on a total of 21 animals All produced prolongation of skin graft survival survival being from 5 to 12 days longer than that seen in control groups of animals treated either with gamma globulin from animals immunized with control supernatants or normal rabbit gamma globulin

Britta Wahren & D Metcalf State Bacteriological Laboratory Stockholm Sweden and Walter and Eliza Hall Institute Melbourne Australia IN VITRO CYTOTOXICITY AGAINST SYNGENEIC EMBRYONIC CELLS BY LYMPHOID CELLS FROM MICE OF HIGH LEUKAEMIC STRAINS

AKR preleukaemic lymphoid cells were found to be cytotoxic to syngeneic embryo and thymus cells in tissue culture This reactivity was detectable with cells from mice aged 3 weeks up to 9 months but was not present with cells from younger mice Cytotoxic reactions to syngeneic tissues were also seen with lymphoid cells from a Moloney virus-carrying C3H strain Lymphoid cells from leukaemic mice did not show this reactivity Similar reactions were not found with syngeneic lymphoid cells of low leukaemia mouse strains Two explanations may be possible (a) virus directed structural difference between aggressor cells and embryonic fibroblasts or (b) an acquired cellular immunity during the preleukaemic period Aquired immune reactivity to the G antigen appeared more likely on the basis of the failure of tumor C+ cells to cause cytotoxic lesions the dispensability of phytohaem agglutination in the reaction and the inhibition of cytotoxicity by incubation of target cells with an antiserum directed to AKR C+ tissue

S Freiesleben Sorensen & F Kissmeyer Nielsen Rigshospitalet Medical Department P and The Institute for Experimental Immunology University of Copenhagen and Blood Bank and Blood Grouping Laboratory Municipal Hospital Arhus Denmark THE MIXED LEUCOCYTE CULTURE TECHNIQUE AS A HISTOCOMPATIBILITY TEST

In the mixed leucocyte culture technique (MLC) recipient lymphocytes are mixed with inactivated donor lymphocytes If the two cell donors are genetically dif

ferent the recipient lymphocytes are stimulated to blastogenesis and cell division It has been shown that reactivity in MLC is mainly controlled by HL-A determined antigens

A method of culture has been worked out which offers good cell survival and early occurrence of significant thymidine incorporation in the stimulated cultures Peak response was shown not only to be determined by the antigen concentration and the sensitivity of the cells but also by the culture conditions The initial response was much less influenced by culture conditions and therefore a better measure of the cellular response

Siblings and parents in 3 families in whom HL A chromosome determinations in the basis of serological leucocyte typing was possible were studied It was predicted that cell donors differing from the responder by two HL A chromosomes should cause a greater stimulation than cell donors differing by only one chromosome In 10 out of 11 experiments two chromosome differing mixtures gave significantly higher responses than one chromosome differing mixtures when measured at day 3 or 4

Anja Tiilikainen M Virolainen P Hayry B Kuhlbäck B Lindström & R Pattonen Department of Serology and Bacteriology and Third Department of Pathology University of Helsinki and Fourth Medical Clinic and Fourth Clinic of Surgery University Central Hospital Helsinki Finland ONE WAY STIMULATION IN MIXED LEUCOCYTE CULTURES STUDIED AFTER RENAL TRANSPLANTATION

Mixed leucocyte culture (MLC) tests were performed according to the one way stimulation procedure in which stimulating cells are treated with mitomycin C. Approximately 85 per cent of the cultures from healthy individuals responded to the antigenic stimulus of leucocytes from a non related individual

Pre transplantation experiments generally correlated well with matching based on leucocyte antigens (cytotoxicity) In MLC tests performed after transplantation the recipients leucocytes responded both to PHA and to mitomycin treated third party leucocytes but the response was weaker than before transplantation No stimulation by the donor s cells was detectable in spite of a poor match in the leucocyte antigens This was true even during rejection The modification of the immune response seemed to occur rapidly after transplantation and adoption of the immunosuppressive treatment in MLC tests cadaveric donor cells maintained in non mixed culture for one day did not stimulate recipient s cells collected one day after transplantation PHA stimulation was affected as early

F Thorsby & F Kissmeyer Nielsen Blood Bank and Dept of Immunohaematology Ullevål Hospital Oslo and Blood Bank and Blood Grouping Laboratory Municipal Hospital Arhus HUMAN TRANSPLANTATION ANTIGENS THE HL A SYSTEM

Human leucocytes contain transplantation antigens skin grafting gives rise to antibodies against donor leucocytes By means of lymphocyte typing skin graft donor recipient pairs were selected for production of HL-A typing antisera of desired specificity The ensuing antibodies were found to be directed against antigens of the LA and 4" sub loci and the specificity showed close correlation to the HL A incompatibility between donor and recipient It was also possible to produce antibodies against new antigens of the HL A system

By means of cytotoxic antisera obtained in this way and antisera from multiparous women the following HL A antigens could be identified
LA sub locus HL A1 HL A2 Ba HL A3 II N LA BS (Da 17 11[1])
4 sub locus HL A5 T12 HF HL A7 HL-A8 4C FJH (BB I ND)
The respective genetic determinants behave as alleles in population and family studies If the frequencies of all mentioned antigens are taken into account about 5 per cent of the possible alleles at the LA sub locus and about 8 per cent of the possible alleles at the 4 sub locus remain to be identified Recent results in our laboratories also indicate that a *third* sub locus closely linked to the HL-A region may exist

HL A typing also seems to permit selection of compatible donor recipient pairs for clinical kidney transplantation with a most favourable influence upon the clinical outcome This is most evident in the cases where donor and recipient have identical HL A antigens all these cases have had minimal rejection episodes and the renal function and general condition is normal On the contrary all kidneys have been rejected in cases where antibodies against donor cells could be detected prior to grafting

A Svejgaard F Kissmeyer Nielsen S Ahrons S Freiesleben Sorensen & F Jørgensen The Blood Bank and Blood Grouping Laboratory the University Hospital Arhus and the Institute for Experimental Immunology University of Copenhagen COMPLEXITY OF THE HL A SYSTEM

The human transplantation system HL A has been clarified by serological investigations mixed leucocyte culture tests (MLC) and homotransplantations The concept of HL A reached by the serological approach depends on the interpretation of the antibody antigen relationship A simplex complex model (simple antibody — complex antigen) of this relationships leads to the assumption of multiple HL A subloci or sites while a complex simplex model tends to decrease the number of subloci The demonstration of cross reactive HL-A iso antibodies—which have been formed even in individuals sensitized with only one of the corresponding antigens—indicates that a complex simplex (or a complex complex) system is involved Based on this assumption two series of mutually exclusive HL A genes have been defined The presently known genes within these series may be combined in 72 different haplotypes making 2628 genotypes possible

It has been possible to find unrelated individuals carrying identical HL A antigens from the two series mentioned Three such unrelated pairs have been tested with MLC to investigate the degree of compatibility In all six directions tested stimulation was observed as compared with non stimulating HL A identical siblings However these unrelated individuals stimulated each other definitely less than related individuals differing in only one HL A haplotype This indicates a rather high degree of compatibility and the survival for 17 days of a skin graft exchanged within one of the pairs confirms this assumption Still the fact that stimulation was obtained indicates that the complexity of the HL A system exceed that of the two series The most likely explanations for this furher complexity are (1) some of the HL A genes known at present as entities may represent two or more different genes (2) one or more additional series of mutually exclusive HL A genes exist

B Mogensen & F Kissmeyer Nielsen The Cancer Research Institute the Danish Cancer Society Radiumstationen and the Blood Bank and Blood Grouping Laboratory Arhus kommunehospital Arhus Denmark THE PROGNOSTIC APPLICATION OF HL A AND ABO TYPING IN PLACENTAL CHORIOCARCINOMA

A foetus suffering from placental choriocarcinoma is rarely born alive at term On the other hand the prognosis of the woman in question who may be regarded as the host for a malignant allogeneic transplant seems unpredictable

The clinical course of the disease in about 90 patients and HL A and ABO typing of 46 of these patients and their husbands and children have revealed much evidence in support of the assumption of a relationship between histocompatibility and the prognosis of placental choriocarcinoma viz that incompatibility between the tumour and its host should give a good prognosis and compatibility a bad one As a consequence of this assumption we propose that the following procedures be undertaken in prospective cases of choriocarcinoma

1 ABO and HL A typing of the patient and her husband

2 ABO and HL A typing of children born in the marriage in question

3 Investigations in the patient for agglutinating or cytotoxic antibodies active against leucocytes or lymphocyte from the husband and the children

Anja Tiilikainen & Helena Kauranen Department of Serology and Bacteriology University of Helsinki Helsinki Finland ANTI LYMPHOCYTE ANTIBODIES AND TOXAEMIA OF PREGNANCY

Although extensive studies of the effect of maternal leucocyte antibodies on the foetus have previously been carried out immunity of the mother has not definitely been shown to harm the foetus

We studied the possibility that the production of leucocyte antibodies would increase maternal hazards A series of blood samples was collected from toxaemic and normal mothers as toxaemia of pregnancy is the condition most likely to follow an insufficient elimination of antigen antibody complexe Some samples were drawn before others 1–30 days after the delivery

The sera were examined using 20 randomly selected lymphocyte preparations in a sensitive modification of the cytotoxicity assay By this means cytotoxic antibodies were found more frequently in toxaemia than in normal pregnant women The difference was quite evident in the primiparous group In the multiparous group however the difference was slight and in part this may be because many women now listed under normal multiparae had suffered from toxaemia in an earlier pregnancy In nonparous control subjects the frequency of cytotoxic antibodies was negligible

Further ways of assaying the correlation between toxaemia of pregnancy and histocompatibility factors will be reported

C Nicole Muller Berat A Salai & R Moulias State Serum Institute Copenhagen and CHU la Pitié Salpetrière Paris France ANTI LYMPHOCYTE SERUM AND HETEROGRAFTS

Skin was grafted in various xenogeneic combinations The purpose of the experiments was threefold

1 To check the role of ALS in suppressing the xenogeneic barrier in our system

2 To analyse the possible various degrees of xenogeneic incompatibility between man and other species

3 To assess whether the administration of ALS directed not against the recipient but against the donor s lymphocytes would exert cytotoxic or enhancing effect

With a view to this DBA 2 kin was grafted to BN rats and vice versa So far human skin was grafted to mice rats and guinea pigs In any one set of experiments skin from the same human subject was used

The results are

1 ALS is able to prolong skin heterografts in the same fashion as homografts namely with the same requirements of timing

2 The survival time of the human skin is longer in mice than in rats and in guinea pigs

3 Anti mouse ALS given to rats carrying mouse skin was reproducibly cytotoxic leading to a hyperacute rejection A determining factor of this reaction is the pre grafting injection

J I Sparck Statens Seruminstitut Copenhagen Denmark GROWTH OF TRANSPLANTED TUMOURS IN MICE TREATED WITH ANTILYMPHOCYTE SERUM

Findings are reported which show that the growth of transplanted tumours is modified when the recipients are treated with antilymphocyte serum

Groups of C3H mice either untreated or treated by intraperitoneal injections of normal rabbit serum or rabbit anti mouse lymphocyte serum (ALS) received grafts (as cell suspension) of primary spontaneous mammary tumours from C3H or DBA donors

As regards the allogeneic transplantation (DBAC3H) the tumour growth was und to be promoted in the groups treated with ALS since the regression of the compatible tumour was delayed or completely prevented

This promotion of tumour growth was only found if donor and host were gene :ally non identical When syngeneic (C3H) spontaneous mammary tumours were afted to C3H mice, there was an inverse effect of immunosuppression of the cipient Here the tumour growth was reduced in the groups treated with ALS

ann i Start King Gustav V Research Institute Stockholm Sweden EXPERIMENTAL PRODUCTION OF HAEMAGGLUTINATING MACROGLOBULINS IN ANIMALS

[illegible] have been elaborated for experimental production of haemagglutinating ma r gl bulins The macroglobulins were obtained 1) by injections of bacteria nt animals in the first p ace Diplo treptococcus agalactiae 2) by injections of the antib dy to RF into animals

It wa f und p ssible t provoke haemagglutinating macroglobulins with b th these methods

As to the first method a su pension in s line f Diplostreptococcus agalactiae was injected repeatedly intraperitoneally (or in another way) into white rats (type Sprague Dawl y)

The second method included everal stag s Fir t the Rheumatoid factor wa i olated from Rheumatoid arthritis serum according to a method described a o in Acta Medica Scand 1957 and J A M A 1965

The isolated RF was injected into rabbits in the way commonly for produc ng antibodies An anti RF serum with high titre was usually obtained The anti RF

serum was injected into white rats A haemagglutinating macroglobulin of RF type mostly appeared after 3 weeks

The above mentioned findings with anti RF serum gave rise to trials of isolating the active RF neutralizing fraction from anti RF serum The RF neutralizing fraction was found to be a 7S globulin (γG or IgG) showing properties other than the common γG in human gammaglobulin

By injecting the 7S globulin in question into rats a haemagglutinating macroglobulin similar or identical with the Rheumatoid factor was obtained

O J Mellbye & J B Natvig Research Institute of Rheumatology Rikshospitalet and Oslo Sanitetsforenings University Hospitals Oslo Norway ANTIBODIES TO PEPSIN TREATED γ GLOBULIN IN SYNOVIAL FLUIDS

Previous work has shown that rheumatoid factor may be produced and consumed *in joints of patients* with rheumatoid arthritis The present study was performed to see whether this was true also for the anti γ globulin antibody to pepsin treated γG globulin the so called pepsin agglutinator (PA) The amount of PA was measured in synovial fluid and serum from 70 patients with rheumatoid arthritis and PA was found in 61 cases In 57 of these the amount in synovial fluid was equal to or somewhat lower than in serum The ratio of the amount of PA in synovial fluid to that in serum was approximately identical to the corresponding ratios of other γG and γM antibodies This was consistent wiht a systemic production of PA followed by a transfer from serum to synovial fluid as in cases of other antibodies In two patients this ratio of PA was higher than that of the other antibodies consistent with a local production of PA in the synovial tissue In two other patients this ratio of PA was lower than that of the other antibodies consistent with a consumption or inhibition of PA in the joints In the latter four patients the ratio of the concentration of C3 (β_1c/β_1a) in synovial fluid to that in serum did not differ from the same ratio in the other patients In all groups of patients this ratio was lower than the corresponding ratio of γM globulin indicating that a local complement consuming process had taken place

The study demonstrated that there are similarities between rheumatoid factor and PA with respect to their occurrence in synovial fluid

E Munthe Research Institute of Rheumatology Rikshospitalet and Oslo Sanitetsforening University Oslo Norway IMMUNOFLUORESCENT STUDIES ON RHEUMATOID TISSUE

Synovial membranes from 18 seropositive and 1 seronegative patients with rheumatoid arthritis were studied in frozen sections using fluorescein labelled antibodies to γG γM and γA globulin β_1C fibrinogen and albumin respectively In all γG γM and β_1C were demonstrated γG sometimes in large quantities The γ globulin were located to plasmacells and to numerous granules of different sizes found intra and extracellularly both in the lining and sublining layer Since the granules also contained complement they probably represented immune complexes β_1C was also found intracellularly in germinal centres together with γ globulins suggesting a local production Marked superficial imbibition with fibrinogen and some albumin were regularly demonstrated

Rheumatoid factor (RF) detected by its ability to bind fluorescein labelled aggregated γG globulin was found in almost all seropositive and in one seronegative patient It was located to plasmacells granules or globular deposit The localiza

tions of RF and β_1C were not identical In 9 cases labelled pepsin treated γG globulin was b und to the tissues indicating antibodies to pepsin treated γ globulin

Two rheumatoid nodules contained large amounts of immunoglobulins β_1C and RF Control synovial membranes from normal subjects as well as from patients with osteoarthritis and pigmented villonodular synovitis did not contain detectable amounts of γ globulins β_1C or anti γ globulin antibodies

Synovial membranes and nodules from 20 of the patients were homogenized washed 10 times and lyophilized Saline eluates were obtained at 56 C for 30 min All eluates from 13 seropositive patients contained RF and some also antibodies to pepsin treated γ globulin Eluates from 7 seronegative patients were negative All eluates from seropositi e as well as seronegative patients contained considerable amounts of γ globulin present partly in an aggregated state

O Tonder & E J Milde Department of Microbiology The Gade Institute University of Bergen School of Medi ine Bergen Norway SEROLOGICAL REACTIONS WITH RHEUMATOID TISSUE

Rheumatoid factor was demonstrated in synovial tissue from patients with rheumatoid arthritis using the technique of mixed agglutination with tissue sections Red cells sensitized with the corresponding antibodies of human or rabbit origin served at indicator cells Among 91 patients with definite rheumatoid arthritis tissue from 90 ad orbed the sensitized cells The mixed agglutination was inhibited by denatured γG globulin by antibodies to γM and by mercaptoethanol indicating that the reaction depended on rheumatoid factor bound in the tissue Apparently there was no definite relationship between the strength of the reaction with tissue and titre of rheumatoid factor in serum Notably tissue from some patients reacted strongl while rheumatoid factor was not demonstrable in serum

Tissue from many patients also gave a positive reacti n with rheumatoid factor applied to the tissue indicating that the reactant for rheumatoid factor is bound in the tissue Heating the sections at 56 C in saline abolished the reactions but treatment of such sections with eluates of rheumatoid synovial tissue re established the reactivity Rheumatoid factor and γG globulin were found in eluates of tissue The γG globulins combined easily with any rheumatoid tissue On the other hand no reaction was obtained with normal or control tissues Accordingly these γG globulins behave as antibodies to something (x) in rheumatoid tissue and presumably rheumatoid factor is bound to the complexes γG/tissue (x)

Pet r Lagercrantz Sten Hammarström Peter Perlm nn & Bengt E Gustafsson Wenner Gren Institute for Experimental Biology University of Stockholm and the Department of Germfree Research Karolinsl a Institute Stockholm Sweden AUTOANTIBODIES TO COLON IN GERMFREE RATS MONOCONTAMINATED WITH *CLOSTRIDIUM DIFFICILE*

Germfree rats monocontaminated with the anaerobic microorganisms *Clostridium difficile* or another Clostridium species (Strain Gf?) produce auto antibodies to colon antigen The antigen can be extracted with phenol water from the faeces of germfree rats Antibodies demonstrable by means of passive haemagglutination of antigen sensitized sheep erythrocytes appear after monocontamination for 35 days or longer The indirect immunofluorescence techniques applied to sections of germfree rat colon gave positive mucosal staining The staining was similar to

that obtained with sera from patients with ulcerative colitis or from rats immunized with rabbit colon No antibodies were found in the sera of germfree rats germfree rats monocontaminated with various other bacteria conventional rats of germfree origin or conventional Sprague Dawley rats

Although the anti colon antibodies of the Clostridium infected rats reacted with the same faeces extract as the antibodies of ulcerative colitis patients or of rabbit colon immunized rats their specificity was different While the latter cross react with polysaccharide from *E coli* 014 those from the Clostridium infected ex germ free rats did not Rats monocontaminated with *Cl difficile* also developed anti bodies to this organism but no cross reaction between *Cl difficile* antigen and colon antigen could be demonstrated This speaks against breakage of tolerance by cross reacting bacterial antogen as the cause of autoimmunity in these rats

T Hjort Institute of Medical Microbiology University of Arhus Arhus Denmark
CHARACTERIZATION OF THYROGLOBULIN AUTO ANTIBODIES BY MEANS OF A HAEMAGGLUTINATION INHIBITION TECHNIQUE WITH ANTIBODY COATED CELLS

Human sera which in a haemagglutination test with thyroglobulin coated cells revealed thyroglobulin antibody titres of 4^5 or more were tested by a haemagglutination inhibition technique with cells coated with highly purified rabbit antibody to human thyroglobulin Theoretical considerations indicated that the titres obtained in this inhibition system would be influenced not only by the concentration of the thyroglobulin antibody but also by its avidity Therefore it should be possible to disclose differences in antibody avidity by comparing the inhibiting and agglutinating activity of the sera

Based on these assumptions sera from 24 patients with lymphadenoid goitre were found generally to contain highly avid thyroglobulin antibodies although a decrease in avidity could apparently occur during the disease Similar findings were made with sera from 23 patients with primary myxoedema A completely different reaction pattern appeared in diseases in which high levels of thyroglobulin antibodies are rarely seen Thus antibodies of very low avidity were found in 5 out of 7 cases with a clinical diagnosis of non toxic goitre (or in all 5 cases of histologically verified non toxic goitre) in 2 out of 3 patients with cancer of the thyroid and in 2 patients with subacute thyroiditis These differences in reactivity of the thyroglobulin antibodies may refer to different pathogenic mechanisms eliciting the autosensitization

J Wasserman Th Packalen I Perlmann C Hedvig Perlman The Central Bacteriological Laboratory of Stockholm City Stockholm and Wenner Gren Institute Stockholm Sweden
ANTIBODY MEDIATED CYTOTOXIC EFFECTS OF NORMAL GUINEA PIG SPLEEN CELLS ON CHICKEN ERYTHROCYTES SENSITIZED WITH GUINEA PIG THYROGLOBULIN

Guinea pigs were injected once with homologous thyroglobulin in complete Freund's adjuvant These animals developed delayed hypersensitivity and haemagglutinating antibodies to thyroglobulin as well as lesions in the thyroid gland In the presence of heat inactivated serum from thyroglobulin immunized guinea pigs spleen cells from normal guinea pigs lysed ^{51}Cr labelled chicken erythrocytes coated with thyroglobulin The reaction was slow commencing after 5 hours and approaching completion after 10 hours after incubation Addition of complement was not necessary

The reaction was specific and required direct contact between spleen cells and antigen coated erythrocytes No cytotoxicity was observed when immune serum was substituted by normal serum or normal spleen cells by an excess of chicken erythrocytes The cytotoxicity of column purified spleen cells was reduced to some extent The antibody mediated cytotoxicity of spleen cells was inhibited by Antimycin A and rabbit anti guinea pig gammaglobulin serum

I Heron Aalborg County and Municipal Hospitals 9000 Aalborg Denmark IMMUNOFLUORESCENCE STUDIES OF RENAL LESIONS

In earlier published papers on kidney biopsies examined by immunofluorescence microscopy a freezing procedure has been used for fixation and cutting of the tissue In this study the method of Sainte-Marie s including fixation in pre cooled ethanol and paraffin embedding was used for routine histology and immuno fluorescence microscopic examination The results of 35 renal biopsies and 9 autopsy kidney specimens from patients suffering from various types of renal lesions are briefly mentioned

The advantages and errors of this immunohistological procedure are discussed Normal renal tis ue was examined with a view to comparing the two methods The renal tissue was quick frozen and sectioned for Coons fluorescence antibody method The spare frozen tissue was allowed to thaw and treated by the method of Sainte Marie s for immunofluorescence microscopy

J Leikola State Serum Institute Helsinki Finland EXPERIMENTALLY INDUCED MONONUCLEOSIS LIKE HETEROPHILE ANTIBODIES IN MAN

The serological diagnosis of infectious mononucleosis is based on the demonstration of the heterophile antibody in the sera of the patients It has been considered to represent a nonspecific response although it is a question of a serologically well defined antibody

Nine human volunteers were immunized with sheep erythrocytes Up to 1/8 of the antibodies formed after the primary stimulation resembled the mononucleosis antibody they agglutinated sheep horse and papain treated ox cells their antigenic receptors on sheep and horse red cells were papain sensitive they were absorbable with ox red cells but not with guinea pig kidney they were macroglobulins No corresponding antibodies were found in rabbit or guinea pig immune sera The observation suggests that the mononucleosis receptor on sheep red cells is immunogenic for man and that the heterophile antibody thus could be formed as a result of a distinct immunogenic stimulus Whether the immunogen is related to the causative agent or whether the response reflects a remarkable specific auto immune process remains unclear

E Nordenfelt & L Kjellén Institute for Medical Microbiology Lund Sweden AUSTRALIAN ANTIGEN IN A SWEDISH HEPATITIS SERIES

In 1965 *Blumberg et al* reported on a new serum protein (Australian antigen) found during a search for isoprecipitins in human sera Australian antigen (Au) is rare in normal american populations It is found in high frequencies in certain diseases especially hepatitis *Recently* the group has shown Au antigen to be associated with a particle of ~ 200 Å

Independently Prince has in a similar isoprecipitin system shown that patients

before they develop serumhepatitis have a specific antigen in their sera This SH antigen has been reported to be identical to Au antigen

The presence of Au antigen in sera of patients with hepatitis has been studied. Reagents have generously been given by dr *A Prince* N Y and Dr *B Blumberg* Philadelphia

Method Geldiffusion according to Ouchterlony

Results The occurrence of australian antigen before and during the early phase of hepatitis was demonstrated

H Nordlinger & U Stenram Department of Pathology University of Uppsala Uppsala Sweden WASTING IN CYCLOPHOSPHAMIDE TREATED RATS

In experiments on the therapeutic effect of a single dose of 110 mg of cyclophosphamide/kg body weight on a transplantable SV 40 tumour in rat it was found that most of the animals cured from their tumours died in a wasting disease after a few months It was later found that also rats that had not been inoculated with tumour cells died in wasting disease following treatment with cyclophorphamide In the terminal wasting stage the rats showed the impaired hair growth hunched posture and high stepping gait described as characteristic of runting

4–5 week old albine mice given 575 mg of cyclophosphamide/kg body weight, died after a few days Mice given 500 mg/kg or lower dose died only occasional in wasting disease

Lungs kidneys and spleen of rats in the final stage of wasting have no bacterial growths

A systematic morphological examination of the syndrome has been started Three days after a single treatment of *3 week old rats with 110 mg of cyclophosphamide/kg* body weight there was a significant decrease in peripheral white blood cell counts and in the weights of the spleen and thymus After 21 days the weights of the spleen and thymus were restored to normal Peripheral white blood cell counts were almost normal The histology of some organs were described

Compared with 3 week old control rats the ability of rats in the wasting stage to reject tumour cells was considerably increased but if compared with controls of the same age it seemed to be decreased

Acta path microbiol s and 77 353-368 1969

Ullevål Hospital Department of Pathology University of Oslo
Oslo Norway

INCORPORATION OF ³H URIDINE INTO THE ADRENAL CORTEX OF MICE AFTER STIMULATION AND INHIBITION

An Autoradiographic Study

By

KÅRE MOLNE

Received 14 iv 69

Histological studies have shown that the various zones of the adrenal cortex react differently to ACTH stimulation (see 10 and 11 for reviews) and biochemical investigations have indicated that the zones produce different steroids (1 9 19 20 21 35 36) However there is still some uncertainty as regards the differential function of the zones and their dependency upon pituitary function This applies particularly to the *zona glomerulosa* and the *zona reticularis*

The *zona glomerulosa* which secretes aldosterone is thought to be largely independent of pituitary function (11) However ACTH induces moderate changes also in the *zona glomerulosa* These changes involve zonal width and lipid content of the cells as well as nuclear and nucleolar size (10 25 28 38)

The role of the *zona reticularis* (which in mice is represented by the inner part of the *zona fasciculata*) is also somewhat uncertain Adherents of the old cell migration theory considered the *zona reticularis* as a senescent part of the adrenal cortex (2 3) After migration from the peripheral parts of the adrenal cortex the cells were supposed to disintegrate near the medullary border Recent studies with radioactive labelling have confirmed that there is a certain displacement of cells in this direction (7 12 18) However studies by *Symington et al* (20 37)have indicated that the *zona reticularis* also is an active and hormone producing part of the adrenal cortex Finally

Requests for reprints should be adressed to Ullevål Hospital Department of Pathology University of Oslo Oslo

I am indebted to Dr *Kristian Fredrik Jenell* Department of Physiology and Biochemistry Dental Faculty University of Oslo for valuable criticism and suggestions and to the head of the Life Insurance Companies Institute for Medical Statistics at the Oslo City Hospitals Dr *Knut Westlund* for his helpful advi e regarding the statistical analyses

Dr *Georg Brabrand* has performed the hypophysectomies His cooperation is greatly appreciated

Tonutti et al (38) have presented the theory of the transformation fields of the adrenal cortex according to which both the *zona reticularis*, and the *zona glomerulosa* act as reserve zones for the *zona fasciculata*.

Since the RNA metabolism may reflect the functional activity of cells and since autoradiography with tritiated RNA precursors may give information about topographical differences in RNA metabolism it was decided to apply this technique to study the various zones of the adrenal cortex in mice. Experiments will be presented below which show the incorporation of tritiated RNA precursors into the adrenal cortex of untreated mice and of mice subjected to various types of adrenocortical stimulation and inhibition.

MATERIAL AND METHODS

Adult male mice of the C57Bl strain were used in this study. The animals were kept under standard housing and dietary conditions as described elsewhere (27). The number and age of the animals in the various experimental groups are given in Table 1.

TABLE 1

Age, Body Weight and Number of Animals in the Various Experimental Groups

	Experimental group	Number	Age (days)	Body weight (g)
1	Untreated animals	27	75–80	22.0–26.5
2	ACTH treated animals	5	79	23.0–26.0
	Controls	5	80	23.5–27.0
3	Formalin stressed animals	5	78–80	20.5–25.0
	Controls	5	78–80	21.5–27.5
4	Dexamethasone treated animals	5	79–80	23.0–25.0
	Controls	5	78–80	21.0–26.0
5	Hypophysectomized animals	5	52–53	17.5–23.0
	Controls	4	51–57	17.0–26.0

Studied at 7 intervals after injection of the isotope.

The material consists of the following groups:

1. *Untreated animals.* Twenty seven animals were injected with ³H uridine and killed at intervals varying from 10 minutes to 24 hours after the injection as shown in Tables 2, 3 and 4.

2. *ACTH treated animals.* Five animals were given 0.6 I.U. of ACTH (0.01 ml Jaton prolongatum A.L.) subcutaneously 3 times a day for 5 days. The animals were killed on the 6th day 1 hour after a final injection of 0.6 I.U. ACTH. ³H uridine was given to this group and to 5 control animals twenty minutes before sacrifice.

3. *Formalin stressed animals.* In order to study the effect of increased endogenous ACTH secretion, a group of animals was subjected to formalin stress. 0.05 ml of a 4 per cent formalin solution was given subcutaneously at 4 hour intervals for 26 hours. The animals were killed 2 hours after the last injection. One hour before sacrifice these animals and a comparable group of control animals were given ³H uridine. It has been shown by *Solem* (34) that the increase in plasma corticosterone after injection of 0.05 ml of 4 per cent formalin in mice is maximal after 2 hours and lasts for 5 hours.

4. *Dexamethasone treated animals.* Dexamethasone was used as depressor of endogenous ACTH secretion. Five animals were given 0.2 mg of dexamethasone

(0 05 ml of Decadron MSD) ubcutaneously twice daily for 5 days The animals were killed on the 6th day 2 hours after a final injection of 0 2 mg of dexametha sone Twenty minutes before sacrifice these animals and a group of control animals were given ^{3}H uridine

5 Hypophysectomized animals Hypophysectomy was performed on 4 animals with a technique which has been described previously (4) These animals and a group of sham operated control animals were killed 41–59 hours after the opera tion ^{3}H uridine was given twenty minutes before sacrifice In order to check the effect of the operation the level of plasma corticosterone was determined at the time of sacrifice as described by *Solem & Brinck-Johnsen* (33) The animals were roused around in the cages during the last 30 minutes before death in order to stimulate a potential ACTH secretion After sacrifice the *sella turcica* was examined under the dissection microscope for remnants of the pituitary glands

Isotopes ^{3}H uridine was used as a precursor of RNA The isotope was obtained from the Radiochemical Centre Amersham England. Each animal received 5 μCi/g body weight intraperitoneally Two batches of isotopes were used which had specific a tivities of 5 0 Ci/mM and 30 Ci/mM respectively The first batch was used in groups 1 4 and 5 and the second in groups 2 and 3

Fixation te hnique and autoradiographic procedure The adrenals were dissected free from fat tissue under a dis ection microscope fixed for 1 day in *Baker*s form aldehyde solution and embedded in paraffin Sections were then cut at 3 microns through the middle of the glands parallell to the longitudinal axis Two or three sections were placed on each slide The slides were deparaffinized covered with Kodak NTB 2 emulsion and exposed for 77 to 84 days at 4 C After development of the autoradiograms with Kodak Dektol the sections were stained with haema toxylin and eosin All sections in each experimental and control group were pro cessed through the same batch of emulsion and developed simultaneously All steps in histological and autoradiographi procedures were standardized as far as pos sible

Evaluation of autoradiograms Grain counts were made over 3 zones of the adrenal ortex In the *ona glomerulosa* and in the outer and inner part of the *fasciculata* In mice there is no clearly defined *ona reticularis* The *ona fasciculata* was there fore somewhat arbitrary divided into a *ona fasciculata interna* and a *zona fasci culata externa* (28) In the *ona fasciculata externa* the outer 5 layers of cells which form definite columns were counted in the *ona fasciculata interna* the inner 5 layers

A section through the middle of the mouse adrenal gland has the shape of an ellipse Grain counts were made in the two sectors of the adrenal cortex along the shortest diam ter of the ellipse Two adrenal sections from each animal (placed on different slides) were studied As regards groups 1–4 grain counting was made over 10 cells within each zone of the adrenocortical sectors In the case of group 5 grain counting was performed over 12 cells in the first section and over 13 in the second one Grain counts were thus made over a total number of 40 cells in each zone from each animal in groups 1–4 and over 50 cells in group 5

In the *ona fasciculata ext rna* of untreated animals the cell membranes could be readily distinguished and in this zone grain counts were performed both over nucleus and cytoplasm In the other zones the cell membranes were indistinct and grain counts were made only over nuclei

The grain counting was performed under oil immersion Cells selected for grain counting were those in which the nuclear membrane the nucleolus and the grains in the film could be se n simultaneously without changing the focus of the micro scope In this way grain counts were performed only over nuclei which were in close contact with the film and errors due to self absorbtion of the β rays in the tissue were reduced

The diameters of the nucleus were determined with an ocular micrometer The cut surface of the nucleus was mostly elliptical The area was calculated ac ording to the formula $\frac{a \times b}{4}\pi$ where a and b represent the two diameters of the ellipse The number of grains per unit area of the nucleus was designated the nuclear grain density and was expressed as grains/$\mu^2 \times 100$ The cells in the *ona fasci culata externa* are polyhedral or irregularly shaped and the area of the cytoplasm could not be satisfactorily calculated

TABLE 2

Grain Counts in the Nucleus Cytoplasm and whole Cells in the zona fasciculata externa *of C57Bl Mice after Injection of ³H Uridine*

Intervals after injection	No of animals	Nucleus (No of grains) Mean	Range	Cytoplasm (No of grains) Mean	Range	Total cell (No of grains) Mean	Range	Relative cytoplasmic labelling (% of total)
10 min	4	9 98	7 5–12 7	0 73	0 6– 0 8	10 72	8 0–13 5	7
20 min	4	18 77	16 0–22 [illegible]	3 55	2 [illegible]– 4 7	22 39	18 6–26 8	16
40 min	4	[illegible] 40	19 9–24 8	6 47	5 7– 7 1	28 89	2[illegible] 7–30 9	[illegible]
60 min	4	11 84	9 9–13 [illegible]	6 71	[illegible] 3– 7 9	18 55	17 3–19 8	36
3 hrs	3	10 97	8 4–13 8	10 31	8 3–12 8	21 28	16 [illegible]–26 7	48
12 hrs	4	6 48	[illegible] 4– 7 5	21 38	10 9–2[illegible] 6	27 86	16 8 3[illegible] 1	77
24 hrs	4	4 20	3 5– 4 6	32 25	27 1–41 2	3[illegible] 46	30 6–4[illegible] 8	89

Ribonuclease treatment Adrenal sections from 4 animals were *treated with ribo nuclease* according to a method described by *Sandritter et al* (3?) Two animals *were killed 20 minutes* after injection of the isotope and 2 after 24 hours Serial *sections of each* adrenal were *deparaffinized* and divided *into two groups One* group was incubated with RNase 1 mg/ml (Wortington Biochemical Corporation New Jersey USA) in veronal acetate buffer (pH 6 0) at 60 C *for 1 hour The con* trol slides were incubated *with buffer only* The number of nuclei and the total *number of* grains were then determined in 10800 μ from the *zona fasciculata* of the RNase treated and control sections This was the total area which was covered by *a graded* ocular eye piece

Background grains of the autoradiograms The background activity in the *autoradiograms was determined* by counting the number of grains in 10800 μ^2 of the film adjacent to the adrenocortical sectors chosen for grain counting The highest back ground activity was 1 8 grains per 100 μ and the average was much lower Correc tion for background activity was therefore considered necessary only in the ribo nuclease experiment

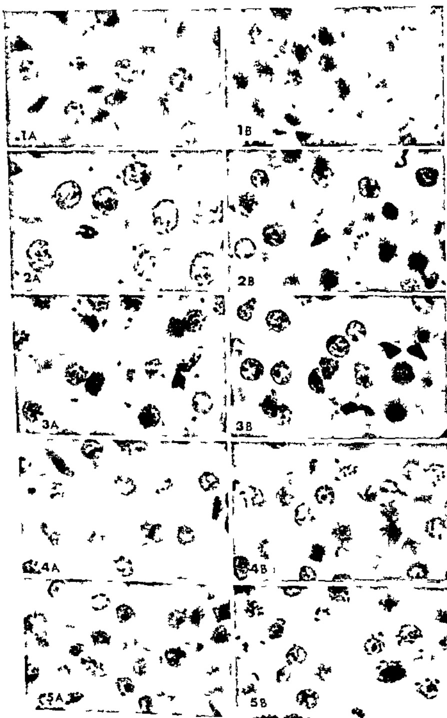
Statistical methods The statistical calculations were based upon mean values in animals and the Wilcoxon Rank Sum Test was used in all analyses The term not significant refers to P values higher than 0 05

RESULTS

Definite labelling of the nucleus and cytoplasm of the adrenocortical cells was seen in all experimental groups after injection of the pre cursor RNase extraction was performed on the adrenal sections from

Figs 1–5

Autoradiograms of the *zona fasciculata externa of mice injected* with labelled RNA precursor H + E × 1100 —*1a* 10 minutes after injection of isotope into normal animals *1b* 24 hours after injection of isotope *2a* Treatment with ACTH for 5 days 20 minutes after injection of ³H uridine *2b* Control section from untreated animal *3a* Formalin stress for 96 hours 1 hour after injection of ³H uridine *3b* Control section from untreated animal *4a* Dexamethasone treatment for 5 days 20 minutes after injection of ³H uridine *4b* Control section from untreated animal *5a* 46 hours after hypophysectomy 20 minutes after injection of ³H uridine *5b* Control section from unoperated animal


1A
1B
2A
2B
3A
3B
4A
4B
5A
5B

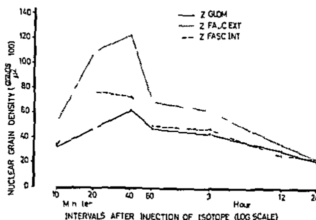


Fig 6

Mean values for nuclear grain density (grains/μ^2 × 100) in the three zones of the adrenal cortex of normal male mice after injection of ^{3}H uridine

animals which were injected with ^{3}H uridine 20 minutes and 24 hours before sacrifice and the treatment removed 92 per cent and 95 per cent of the grains in these two groups It is therefore likely that most of the activity in the autoradiograms was due to radioactive RNA

Incorporation of ^{3}H uridine into Normal Animals

Ten minutes after injection of ^{3}H uridine almost all grains were located over the nuclei in all the zones (Fig 1a) Grain counting over the *zona fasciculata* (Table 2) showed that only 7 per cent of the grains were located over the cytoplasm With increase of the survival periods an increasing number of grains was found over the cytoplasm By 3 hours 52 per cent of the activity was over the nucleus and 48 per cent over the cytoplasm and by 24 hours 89 per cent was over cytoplasm and 11 per cent over nuclei (Fig 1b)

The cell membranes of the adrenocortical cells were frequently indistinct particularly in the *zona glomerulosa* and in the *zona fasciculata interna* and the area of cytoplasm could not be satisfactorily determined Most of the comparisons of activity in the experiments were therefore based on grain counting over nuclei only The nuclei were selected for counting according to pretermined criteria as described above

The *adrenocortical nuclear grain counts* of untreated animals is shown in Tables 3 and 4 and Fig 6 The nuclear grain density increased during the first 20 or 40 minutes after injection of the isotope and then decreased rapidly The decrease was relatively slow after the first hour This pattern was similar for all adrenocortical zones The nuclear grain density reached maximum slightly earlier in the *zona fasciculata interna* than in the other zones (Fig 6 and Table 3) However the dif

TABLE 3

Mean Values for Nuclear Grain Density (Grains/μ^2 × 100) in the 3 Adrenocortical Zones at Different Intervals after Injection of ³H Uridine

Intervals after injection	No of animals	Zona glomerulosa		Zona fasciculata ext		Zona fasciculata int	
		Mean	Range	Mean	Range	Mean	Range
10 min	4	32 91	26 3–40 4	53 94	43 8– 67 3	33 93	25 1–43 2
20 min	4	48 06	34 6–58 6	108 83	90 3–126 5	77 43	66 6–85 3
40 min	4	61 50	56 9–63 4	122 27	107 2–141 0	7[illegible] 78	61 1–88 0
60 min	4	47 64	43 0–51 1	68 28	55 7– 75 0	48 87	44 6–50 7
3 hrs	3	44 22	33 7–54 6	61 97	49 1– 76 8	47 41	33 3–65 1
12 hrs	4	30 07	26 6–34 9	34 57	28 2– 42 6	27 43	19 8–31 1
24 hrs	4	21 91	17 4–28 6	23 18	18 6– 27 6	22 52	19 9–24 1

TABLE 4

Mean Values of Grains per Nucleus in the 3 Adrenocortical Zones at Different Intervals after Injection of ³H Uridine

Intervals after injection	No of animals	Zona glomerulosa		Zona fasciculata ext		Zona fasciculata int.	
		Mean	Range	Mean	Range	Mean	Range
10 min	4	5 32	4 3–6 6	9 98	7 5–12 7	6 69	5 1– 8 1
20 min	4	7 26	5 1–8 4	18 77	16 0–22 2	14 21	12 6–15 7
40 min	4	8 76	8 3–9 1	22 40	19 9–24 8	15 22	13 3–17 0
60 min	4	6 42	5 4–7 1	11 84	9 9 13 5	8 70	8 2– 9 1
3 hrs	3	5 96	5 2–6 6	10 97	8 4–13 8	9 04	6 4–11 9
12 hrs	4	4 49	4 2–5 1	6 48	5 4– 7 5	5 43	4 1– 6 1
24 hrs	4	3 32	2 6–4 2	4 20	3 5– 4 6	4 48	3 8– 4 9

ference was not so apparent when grain counts per nucleus were analysed (Table 4) and it is doubtful whether it reflects a true functional difference between the zones

Although the shape of the incorporation curves were similar in the zones the absolute values were clearly different Thus the activity in the *zona fasciculata externa* was definitely higher than in the two other zones (Tables 3 and 4) There was also a tendency for the *zona fasciculata interna* to have higher incorporation values than the *zona glomerulosa* This applies both to nuclear grain density and to grains per nucleus and probably reflects true differences between the two zones

In the *zona fasciculata externa* the labelling of the cytoplasm was also studied (Table 2) During the first 40 minutes the total number of grains per cell increased At 60 minutes the grain counts showed somewhat lower values and after 3 hours the counts again increased up to 24 hours The biphasic pattern of the incorporation curve will be discussed later

TABLE 5

Mean Values for Nuclear Grain Density (Grains/$\mu^2 \times 100$) in the 3 Adrenocortical Zones after Injection of ^3H Uridine Groups 2 4 and 5 were Killed 20 Minutes after Injection of the Isotope and Group 3 after 60 Minutes

Experimental groups	No of animals	Zona glomerulosa Mean	Zona glomerulosa Range	Zona fasciculata ext Mean	Zona fasciculata ext Range	Zona fasciculata int Mean	Zona fasciculata int Range
2 ACTH							
treatment	5	48 31	33 8–75 1	164 39	129 0–195 5	158 07	111 9 198 5
Controls	5	57 58	34 2–70 9	100 20	78 3–121 3	79 42	56 0 106 0
3 Formalin							
stress†	5	68 27	63 9–79 8	188 43	155 1–205 9	149 94	122 7–171 7
Controls	5	64 63	50 0–81 9	88 32	65 3–113 5	62 89	46 4 90 3
4 Dexamethasone							
treatment§	5	38 81	26 1–51 1	34 80	27 2– 44 6	34 01	25 9 41 9
Controls	5	36 24	29 9–41 4	75 77	63 1– 87 3	59 18	52 1 67.5
5 Hypophy							
sectomy//	4	31 85	20 3–45 1	23 87	13 9– 35 7	20 18	10 6 31 1
Controls	5	39 72	33 9–49 5	79 93	62 8–116 4	63 71	40 8 101 6

TABLE 6

Mean Values of Grains per Nucleus in the 3 Adrenocortical Zones after Injection of ^3H Uridine Groups 2 4 and 5 were Killed 20 Minutes after Injection of the Isotope and Group 3 after 60 Minutes

Experimental groups	No of animals	Zona glomerulosa Mean	Zona glomerulosa Range	Zona fasciculata ext Mean	Zona fasciculata ext Range	Zona fasciculata int Mean	Zona fasciculata int Range
2 ACTH							
treatment	5	8 65	6 1–12 6	39 90	29 3–47 9	35 75	22 9 43 3
Controls	5	8 93	5 3–11 2	18 74	14 5–22 3	15 25	10 8–19 1
3 Formalin							
stress†	5	10 77	10 0 12 8	39 68	33 0–42 7	29 21	23 6 35 2
Controls	5	9 69	6 6–12 1	16 25	12 3–21 7	11 65	9 0–16 1
4 Dexamethasone							
treatment§	5	5 86	4 4– 7 7	5 64	4 8– 7 1	5 22	4 2– 6 0
Controls	5	5 87	4 7– 7 4	13 90	11 5–17 4	11 33	9 6 13 1
5 Hypophy							
sectomy//	4	5 00	3 5– 7 4	4 07	3 0– 6 0	4 32	2 7– 7 0
Controls	5	6 65	5 6– 7 8	16 04	12 9–20 9	12 95	8 2 18 2

0 6 i u s c 3 times daily for 5 days
† 0 05 ml of a 4 per cent solution at 4 hour intervals for 26 hours
§ 0 2 mg s c twice daily for 4 days
// 41–59 hours survival periods

Incorporation of ^3H uridine after Stimulation and Inhibition of the Adrenal Cortex

The results of nuclear grain counting from animals which were subjected to various types of adrenocortical stimulation and inhibition are shown in Tables 5 and 6 and Figs 2–10

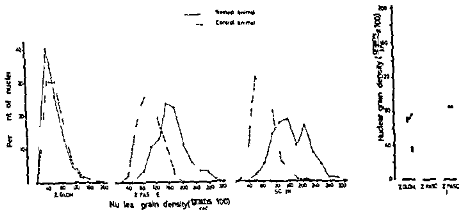


Fig 7

Nuclear grain density (grains/μ^2 × 100) in the adrenal cortex of male mice after ACTH treatment for 5 days The treated animals and the controls were killed 20 minutes after injection of ^{3}H uridine The mean values for each animal are shown to the right The curves to the left are based on the pooled results from the groups

ACTH treatment

After treatment with exogenous ACTH for 5 days the nuclear grain density of the zona *fasciculata externa* and *interna* was clearly higher than in the control animals (p = 0 01 Figs 2 and 7) This difference was even greater when the number of grains per nucleus was analysed (Table 6) Both the nuclear size and the concentration of nuclear grains had thus increased after ACTH treatment The effect on the *zona glomerulosa* was less obvious after ACTH treatment The number of grains per nucleus was unchanged and the nuclear grain density had even decreased slightly as compared with controls This indicates that the nuclear size of the *zona glomerulosa* had increased after long term treatment while the RNA metabolism per nucleus was unchanged as judged by the present technique

Formalin stress is known to be a potent stimulant of corticosterone secretion in mice (34) and the effect upon uridine incorporation was similar to that of exogenous ACTH (Tables 5 and 6 Figs 3 and 8) The effect on the *zona fasciculata externa* and *interna* was even more marked than after ACTH treatment The *relative* increase in activity was somewhat higher in the *zona fasciculata interna* than in the *zona fasciculata externa* both in ACTH and formalin treated animals No change was seen in the *zona glomerulosa* after formalin treatment

Dexamethasone Treatment

Dexamethasone was injected for 5 days to supress the endogenous ACTH secretion Tables 5 and 6 and Figs 4 and 9 demonstrate that the grain counts were reduced both in the *fasciculata externa* and *interna* (P = 0 01) There was no change in nuclear size and the decrease in

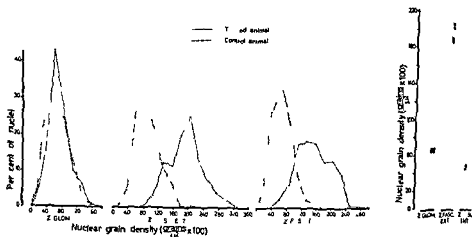


Fig 8

Nuclear grain density (grains/$\mu^2 \times 100$) in the adrenal cortex of male mice after formalin stress for 26 hours The treated animals and the controls were killed 60 minutes after injection of ^3H uridine The mean values for each animal are shown to the right The curves to the left are based on the pooled results from the groups

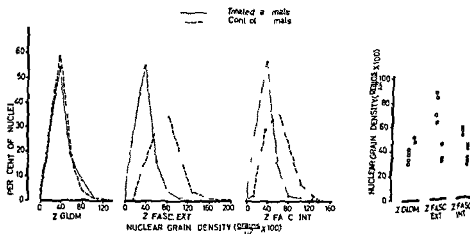


Fig 9

Nuclear grain density (grains/$\mu \times 100$) in the adrenal cortex of male mice after dexamethasone treatment for 5 days The treated animals and the controls were killed 20 minutes after injection of ^3H uridine The mean value for each animal are shown to the right The curves to the left are based on the pooled results from the groups

nuclear grain density and in grain counts per nucleus was therefore of the same magnitude No significant change was found in the zona *glomerulosa*

Hypophysectomy was performed on 4 animals which were killed from 41 to 59 hours after the operation The level of plasma corticosterone was determined by the method of *Solem & Brinck Johnsen* (33) The mean level was 4 1 μg/100 ml (range 0 0–6 7 μg/100 ml) in

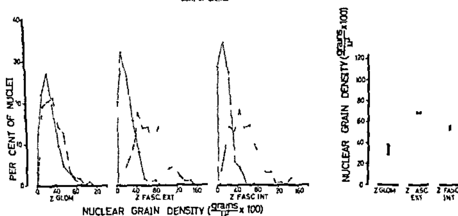


Fig 10

Nuclear grain density (grains/μ × 100) in the adrenal cortex of male mice 41–59 hours after hypophysectomy The operated animals and the controls were killed 90 minutes after injection of ^{3}H uridine The mean values for each animal are shown to the right The curves to the left are based on the pooled results for the group

operated animals and 30 7 μg/100 ml (range 24 0–40 0 μg/100 ml) in the 5 controls The values in the operated animals indicate a cessation of pituitary ACTH secretion (34)

The effect of hypophysectomy on the adrenocortical uridine incorporation was similar to that of dexamethasone treatment (Tables 5 and 6 Figs 5 and 10) The labelling of the *zona fasciculata interna* and *externa* was significantly lower than in the controls (P = 0 02) whereas the incorporation into the *zona glomerulosa* was unchanged

DISCUSSION

The present study indicates that the RNA metabolism is different in the 3 adrenocortical zones and that the various zones also react differently to pituitary stimulation and inhibition Before discussing these results a few comments will be made on the validity and limitations of the technique which was used

It has been shown by others (22) that uridine 5 ^{3}H is a relatively specific precursor of RNA and ribonuclease treatment removed 92–95 per cent of the activity in the sections in the present material Neutral formalin which was used as a fixative extracts completely all free acid soluble nucleotides (13) and does not remove significant amounts of RNA during the first 24 hours (23) It is therefore likely that most of the activity in the sections was bound to ribonucleic acids and that the radioactive RNA was retained in the tissues

The selection of nuclei for counting which was used in this study

allows reliable comparisons between different sections and it also reduces errors due to selfabsorption of β rays (26) in the tissue

All steps in the autoradiographic procedure were standardized as far as possible However considerable variations between groups of slides processed at different times were observed (Table 5) Is was therefore important that all slides from each experimental and control group were processed simultaneously throughout the autoradiographic procedure

Provided that the intra and extra cellular pools of precursors remain of similar magnitude under the various experimental situations (31) dissimilarities in grain counts presumably also indicate true differences in the amount of labelled RNA Increased incorporation of precursor thus probably indicates a raised RNA synthesis Further interpretation of the results in terms of turnover of total RNA or of the different types of RNA must be tentative at best

Table 2 shows that with increasing survival periods a gradual shift of label from the nucleus to the cytoplasm took place Similar observations have been made in many other tissues (*e g* 30) Most probably this shift reflects a movement of newly synthesized RNA

No certain explanation can be given for the biphasic incorporation curve of the cells of the *zona fasciculata externa* (Table 2) A peak was reached at 60 minutes After a transitory decrease the number of grains per cell again increased Findings were similar also in another strain of mice (29) The results of the RNase extractions indicate that both peaks on the incorporation curves represent RNA and not other radioactive substances A possible interpretation of the unexpected shape of the curves may be that they represent the sum of different fractions of radioactive RNA with separate base composition and turn over rates It is interesting however that this type of curve was not found by Oehlert after injection of ^3H cytidine into mice (30)

There was a clear difference in the incorporation of precursors into the various zones of the adrenal cortex of normal mice (Fig 6) This finding thus demonstrate that the zonation reflects true metabolic differences and not only variations in the topographic arrangement of the cells It also shows that there is a true difference between the inner and the outer part of the *zona fasciculata* in mice although the cytologic differences are modest (28) The inner zone presumably corresponds to the *zona reticularis* of other mammals

The highest incorporation was found in the *zona fasciculata externa* and the lowest in the *zona glomerulosa* The *zona fasciculata interna* exhibited an intermediate pattern A similar pattern of distribution was found whether the activity was expressed as grains per nucleus or as grains per unit nuclear area (Tables 3 and 4)

If the pools were the same (34), the cells with the highest incorporation of uridine might be expected also to have the highest RNA content However histochemical and biochemical studies on other

mammals have indicated that the *zona reticularis* has the highest RNA content and the *zona fasciculata* the lowest (8 37) Furthermore the adrenocortical nucleolar size in mice has been measured (28) and the largest nucleoli were found in the *zona fasciculata interna* and the smallest in the *zona glomerulosa* These findings do not quite fit with the results obtained in the present study and no explanation can be given for this apparent discrepancy

All types of stimulation and inhibition of the adrenal cortex gave marked alterations in the labelling of the adrenocortical zones and administration of exogenous hormones (ACTH and dexamethasone) gave results similar to those obtained after endogenous stimulation and inhibition (formalin stress and hypophysectomy)

Generally stimulation of the cortex resulted in a marked increase and inhibition in a decrease in the incorporation of RNA precursors This finding is in accordance with several previous biochemical studies of adrenal glands (5 6 14 15 17 24 37) Recently a few publications have indicated a *decreased* incorporation of RNA precursors after ACTH stimulation (see *e g* 16) No explanation has so far been given for the conflicting biochemical findings

The experiments showed that pituitary stimulation mainly acted on the two zones of the *fasciculata* The *relative* increase was higher in the *zona fasciculata interna* (or *zona reticularis* of other mammals) than in the *zona fasciculata externa* This finding support the concept that the *zona fasciculata* and *reticularis* act as one functional unit (20) and it does not fit with the hypothesis that the *zona reticularis* is a senescent zone (2 3)

Prolonged stimulation with ACTH produced moderate increase in nuclear size in the *zona glumerulosa*, and no change in grain counts per nucleus Neither formalin stress nor inhibition experiments produced any apparent change in nuclear size or labelling of this zone Thus the *zona glomerulosa* is largely but not entirely independent of pituitary stimulation

The difference in the effect upon the *zona glomerulosa* after ACTH treatment and formalin stress may be due to differences in the duration of the experiments (Table 5) Other experiments have demonstrated a moderate increase both in nuclear and nucleolar size (28 38) and in lipid content (10) of the *zona glomerulosa* after prolonged ACTH treatment These findings indicate changes also in the RNA synthesis The condition of the present experiments however may not have been optimal for detecting these small changes

SUMMARY

The object of the present study was to investigate by autoradiography the incorporation of the RNA precursor ^{3}H uridine into the adrenal

12 Diderholm H & Hellman B The cell migration in the adrenal cortex of rats studied with tritiated thymidine Acta physiol scand 50 197–202 1960
13 Edström J E In Methods in Cell Physiology ed D M Prescott Academic Press New York 1 417–447 1964
14 Farese R V Further studies on the stimulation of adrenal protein synthesis by ACTH An effect on microsomes and ribosomes Endocrinology 74 579–585 1964
15 Farese R V & Schnure J J Effects of ACTH on adrenal RNA synthesis Endocrinology 80 872–882 1967
16 Ferguson J J Morita Y & Mendelsohn L Incorporation in vitro of precursor into protein and RNA of rat adrenal glands Endocrinology 80 521–526 1967
17 Fiala S Sproul F E & Fiala A The action of corticotropin (ACTH) on nucleic acids and subcellular elements of the adrenal cortex J Biophysic and Biochem Cytol 2 115 126 1956
18 Ford J K & Young R W Cell proliferation and displacement in the adrenal cortex of young rats injected with tritiated thymidine Anat Rec 146 125–137 1963
19 Giroud C J P Stachenko J & Venning E H Secretion of aldosterone by the zona glomerulosa of rat adrenal glands incubated in vitro Proc Soc Exp Biol and Med (N Y) 92 154–158 1956
20 Griffiths K Grant J K & Symington T A biochemical investigation of the functional zonation of the adrenal cortex in man J Clin Invest 23 776–785 1963
21 Griffiths K & Glick D Determination of the 11 β hydroxylase activity in microgram samples of tissue its quantitative histological distribution in the rat adrenal and the influence of corticotrophin J Endocrin 35 1–12 1966
22 Hayhoe F G J & Quaglino D Autoradiographic investigations of RNA and DNA metabolism of human leucocytes cultures with phytohæmagglutinin uridine 5 3H as a specific precursor of RNA Nature 205 151–154 1965
23 Lagerstedt S The effect of formaldehyde fixation on the amount of ultraviolet absorbing substances released from tissue sections in the histochemical ribonuclease test Z Zellforsch 45 472–482 1957
24 Logan J E Heagy F C & Rossiter R J Phosphorus metabolism of the adrenal gland Effect of hypophysectomy and administration of ACTH on the incorporation of radioactive phosphate into the RNA nucleotides Endocrinology 56 455–460 1955
25 Luse S Fine structure of adrenal cortex In The adrenal cortex By 26 authors Ed A B Eisenstein A Churchill Ltd London 1967 Pp 1–60
26 Maurer W & Primbsch E Grosse der β selbstabsorption bei der 3H autoradiographie Exp Cell Res 33 8–18 1964
27 Molne K The adrenal weight of mice with spontaneous adrenocortical lipid depletion A comparison with untreated, ACTH and Dexamethasone treated C57 Bl mice Acta path microbiol scand 75 37–50 1969
28 Molne K Histometric studies on the adrenal cortex of mice with spontaneous adrenocortical lipid depletion A comparison with dexamethasone and ACTH treated C57Bl mice Acta path microbiol scand 75 51–63 1969
29 Molne K Autoradiographic studies on the incorporation of 3H uridine into the adrenal glands of mice with spontaneous adrenocortical lipid depletion A comparison with C57Bl mice Acta path microbiol scand 77 353–362 1969
30 Oehlert W Autoradiographische Untersuchungen zur Ribonukleinsaure Synthese in der verschiedenen Strukturen der Zelle Beitr path Anat 124 311–350 1961
31 Perry R E In Methods in Cell Physiology Ed D M Prescott Academic Press New York 1 305–326 1964
32 Sandritter W Pilat G & Theiss E Zur Wirkung der Ribonuklease auf Leberzellen Quantitative UV Mikrospektrographische Untersuchungen Exp Cell Res Suppl 4 64–82 1957
33 Solem J H & Brinch Johnsen R An evaluation of a method for determination of free corticosteroids in minute quantities of mouse plasma Scand J Clin Lab Invest Suppl 80 1–4 1965

34 *Solem J H* Plasma corticosteroids in mice With special regard to sex differences in adrenocortical responsiveness to exogenous corticotrophin Scand J Clin Lab Invest Suppl 93 1-36 1966
35 *Stachenko J & Giroud C J P* Functional zonation of the adrenal cortex Pathways of corticosteroid biogenesis Endocrinology 64 730-742 1959
36 *Stachenko J & Giroud C J P* Functional zonation of the adrenal cortex Site of ACTH action Endocrinology 64 743-752 1959
37 *Symington T & Davidson J N* The effect of exogenous ACTH and conditions of stress on the chemical composition of the human adrenal gland Scot med J 1 15-31 1956
38 *Tonutti F Bahner F & Muschke E* Die Veranderungen der Nebennierenrinde der Maus nach Hypophysektomie und nach ACTH Behandlung quantitativ betrachtet am Verhalten der Zellkern volumina Endokrinologie 31 266 284 1954

Acta path microbiol scand 77 369-378 1969

Ullevål Hospital Department of Pathology University of Oslo
Oslo Norway

AUTORADIOGRAPHIC STUDIES ON THE INCORPORATION OF ³H URIDINE INTO THE ADRENAL GLANDS OF MICE WITH SPONTANEOUS ADRENOCORTICAL LIPID DEPLETION

A Comparison with C57Bl Mice

By

KÅRE MOLNE

Received 17 iv 69

In adult mice of the AKR/0 strain and in the substrains AC and CS the adrenal cortex exhibits specific cytological characteristics At the time of sexual maturation a spontaneous depletion of lipids takes place in the adrenocortical cells of both sexes In adult males the adrenal cortex is almost devoid of lipids as judged by the Sudan staining (Figs 1 and 2) In females the depletion is somewhat less pronounced and there is considerable individual variation (1)

Light and electron microscopic studies on mitochondria of adreno cortical cells of mice with spontaneous adrenocortical lipid depletion and of normal mice subjected to stress (2 13) have indicated that the metabolic activity of the adrenal cortex of AKR/0 mice may be higher than that of normal glands It has therefore been suggested that the permanent lipid depletion of the adrenal cortex reflects a state of high activity possibly caused by pituitary hyperfunction (4) However chemical studies on adrenal glands from AKR/0 hybrids have indicated that the corticosterone production is decreased compared with normal animals (14) Furthermore experiments with hypophysectomy and ACTH substitution have shown that the lipid depletion is dependent upon an intact pituitary function but that it can hardly be explained in terms of a simple pituitary hyperfunction (5) Histometric studies on the adrenal cortex of these mice did not indicate an abnormal pituitary activity (11) The functional state of the permanently lipid depleted adrenal cortex is therefore not clear

Requests for reprints should be adressed to Ullevål Hospital Department of Pathology University of Oslo Oslo Norway

I am indebted to the head of the Life Insurance Companies Institute for Medical Statistics at the Oslo City Hospitals dr *Knut Westlund* for valuable discussion of the results



Figs 1–2

Fig 1 Adrenal gland of AC male mouse 12 weeks old Spontaneous adrenocortica lipid depletion Sudan III × 33

Fig 2 Adrenal gland of C57Bl male mouse 12 weeks old Normal adrenocortica lipid pattern Sudan III × 33

A number of biochemical studies have shown that the functiona activity of the adrenal cortex is reflected in the RNA metabolism of the glands (*e g* 6 7 8 15) and recent autoradiographic studies (19) showed that the incorporation of ^3H uridine into the different adreno cortical zones varied markedly with the level of the ACTH stimulation It was therefore thought that similar studies on mice with adreno cortical lipid depletion might give an indication about the adreno-cortical function and possibly also about the pituitary adrenocortical relationship in these animals

MATERIAL AND METHODS

Adult male mice of the AC and C57Bl strains were used in this study The AC strain is a hybrid line derived from the AKR/O strain and is homozygous for the adrenocortical lipid depletion gene (3) The C57Bl strain exhibits a normal pattern of adrenocortical lipids (Figs 1 and 2)

The animals were kept under standard housing and dietary conditions as de scribed elsewhere (10) All animals were between 75 and 80 days of age The mean body weight was 22 5 g (range 19 0–24 5 g) for the AC mice and 24 1 g (range 22 0–26 5 g) for the C57Bl mice

The incorporation of ^3H uridine into the adrenal glands was studied at intervals varying from 10 minutes to 24 hours after injection of the isotope (Tables 1 and 2) Four animals from each strain were injected with isotope in all groups but due to technical errors some of the microscopical slides had to be discarded A few groups therefore consist of only 3 animals The isotope was injected between 8 and 9 A M and all animals with the same survival periods were injected on the same day All experiments were completed within 30 days

Isotope Uridine 5 ^3H (s a 5 Ci/mM) was used as a precursor for RNA (9) The

---

The results of the C57Bl series have been published previously in this journal (12)

isotope was obtained from the Radiochemical Centre Amersham England Each animal received 5 μCi per gram body weight intraperitoneally The same batch of isotope was used for all animals

Fixation technique and autoradiographic procedure The animals were killed by cervical dislocation The adrenals were dissected free under a dissection microscope fixed for 1 day in *Baker's* formaldehyde solution and embedded in gelatine Sections were then cut through the middle of the glands at 3 microns and two sections were mounted on each slide The slides were deparaffinized covered with Kodak NTB 2 emulsion and exposed for 81 days at 4 C After development of the autoradiograms with Kodak Dektol the sections were stained with haematoxylin and eosin. All sections were processed through the same batch of emulsion and developed simultaneously All steps in the histological and autoradiographic procedures were standardized as far as possible

Evaluation of autoradiograms Grain counts were made both over the adrenal cortex and the *medulla* Since the *zona reticularis* is poorly delimited in mice the *zona fasciculata* was arbitrarily divided into a *zona fasciculata externa* and interna (11) Grain counts were then performed in the *zona glomerulosa* and in the *zona fasciculata externa* and *interna* In the *zona fasciculata externa* the outer 5 layers of cells which form definite columns were counted and in the *zona fasciculata interna* the inner 5 layers

A section through the middle of the mouse adrenal gland has the shape of an ellipse Grain counts of the *cortex* were made in the two sectors along the shortest diameters of the ellipse In the *medulla* the grain counts were made along the longest diameter Two adrenal sections from each animal on different slides were studied On both slides grain counts were made over 10 cells on each zone of the two adrenocortical sectors In the *medulla* 20 cells were counted Grain counts were thus made over 40 cells from each adrenocortical zone and over 40 cells in the *medulla*

In the *zona fasciculata externa* the cell membranes could be readily distinguished and in this zone grain counts were performed both over the nucleus and the cytoplasm In the other zones the cell membranes were indistinct and grain counts were therefore made only over nuclei

The diameter of the nucleus was determined with an ocular micrometer The cut surface of the nucleus was usually elliptical and the area was calculated according to the formula $\frac{a \times b}{4} \pi$ where a and b represent the two diameters of the ellipse The number of grains per unit area of the nucleus was designated the nuclear grain density and expressed as grains/μ^2 $\times$ 100 The area of the cytoplasm could not be satisfactorily measured and the grain density per unit area of the cytoplasm could therefore not be calculated

The grain counting was performed under oil immersion Cells selected for grain counting were those in which the nuclear membrane the nucleolus and the grains in the film could be seen simultaneously without changing the focus of the microscope In this way grain counts were performed only over nuclei which were in close contact with the film and errors due to self absorbtion of the β rays in the tissue were minimal

Background grains in the autoradiograms The background activity in the autoradiograms was determined in the film adjacent to the adrenocortical sectors which were chosen for grain counting 10000 μ^2 of the film was scanned in each section The highest background activity was 1 7 grains per 100 μ^2 and on average it was much less No correction for background activity was therefore found necessary in this study

RESULTS

The incorporation of isotope into the adrenals of the two strains at various intervals is shown in Tables 1-3 and in Figs 3-6

In the adrenocortical nuclei of both strains the grain counts reached maximum after 20 or 40 minutes decreased rapidly during the next 30 minutes and then decreased slowly over the following 24 hours (Table 1 and Figs 3-5)

TABLE 1

Nuclear Grain Density (Grains/$\mu^2 \times 100$) in the 3 Adrenocortical Zones and in the Medulla of AC and C57Bl Mice at Different Intervals after Injection of ^3H Uridine

Intervals after injection	Strain	No of animals	Zona glomerulosa Mean	Zona glomerulosa Range	Zona fasciculata ext Mean	Zona fasciculata ext Range	Zona fasciculata int Mean	Zona fasciculata int Range	Medulla Mean	Medulla Range
10 minutes	AC	3	43 54	30 9–50 1	72 69	56 7– 85 0	38 60	27 3–50 7	28 85	22 3–39 3
	C57Bl	4	32 91	26 3–40 4	53 94	43 8– 67 3	33 93	25 1–43 2	29 37	26 1–29 3
20 minutes	AC	4	74 07	65 5–94 9	167 67	145 0–183 0	90 06	83 7–98 8	70 92	54 0–86 0
	C57Bl	4	48 06	34 6–58 6	108 83	90 3–126 5	77 43	66 6–85 3	82 05	63 1–115 2
40 minutes	AC	4	94 61	88 4–99 5	169 46	146 2–188 2	87 29	74 1–102 7	55 48	36 6–77 2
	C57Bl	4	61 50	56 9–63 4	122 27	107 2–141 0	72 78	61 1–88 0	58 42	42 5–74 3
60 minutes	AC	3	83 00	75 2–96 1	125 67	103 8–139 2	78 30	70 7–82 2	39 90	34 5–44 0
	C57Bl	4	47 64	43 0–51 1	68 28	55 7– 75 8	48 87	44 6–50 7	40 06	35 2–50 7
3 hours	AC	4	75 73	66 0–86 8	93 50	85 8–110 3	67 92	58 5–74 4	30 39	22 0–35 4
	C57Bl	3	44 22	33 7–54 6	61 97	49 1– 76 8	47 41	33 3–65 1	31 03	28 6–32 9
12 hours	AC	3	61 01	57 5–66 8	56 77	48 8– 61 4	55 73	52 8–59 2	19 74	18 1–22 8
	C57Bl	4	30 07	26 6–34 9	34 57	28 2– 42 6	27 43	19 8–31 1	16 65	14 1–19 2
24 hours	AC	4	32 43	28 7–35 5	32 96	29 1– 37 1	29 87	23 1–41 9	13 59	11 0–14 9
	C57Bl	4	21 91	17 4–28 0	23 18	18 6– 27 6	22 52	19 9–24 1	12 35	11 1–15 1

TABLE 2

Grain Counts in the Nucleus, Cytoplasm and Whole Cells in the Zona fasciculata externa of AC and C57Bl Mice after Injection of ^3H Uridine

Intervals after injection	Strain	No of animals	Nucleus (No of grains) Mean	Range	Cytoplasm (No of grains) Mean	Range	Total cell (No of grains) Mean	Range	Cytoplasmic labelling (% of total)
10 minutes	AC	3	13.35	10.5–15.0	0.80	0.7–0.9	14.15	11.3–15.9	6
	C57Bl	4	9.38	7.5–12.7	0.73	0.6–0.8	10.7?	8.0–11.5	7
20 minutes	AC	4	32.87	31.0–35.1	4.77	4.0–5.4	37.64	35.0–40.?	13
	C57Bl	4	18.77	16.0–22.2	3.55	2.5–4.7	22.32	18.6–26.8	16
40 minutes	AC	4	30.44	28.6–31.4	6.60	5.9–7.5	37.04	34.5–38.8	18
	C57Bl	4	22.40	19.9–24.8	6.4?	5.7–7.1	28.82	26.7–30.9	22
60 minutes	AC	3	23.29	17.4–28.1	9.24	7.8–11.2	32.52	25.2–39.3	28
	C57Bl	4	11.84	9.9–13.5	6.71	5.3–7.9	18.55	17.3–19.9	36
3 hours	AC	4	17.72	16.1–20.1	15.90	14.4–18.3	33.62	31.2–38.4	47
	C57Bl	3	10.97	8.4–13.8	10.31	8.3–12.8	21.28	16.7–26.7	48
12 hours	AC	3	10.81	8.9–12.0	35.75	28.2–42.5	46.56	37.0–54.2	77
	C57Bl	4	6.48	5.4–7.5	21.38	10.9–27.6	27.86	16.8–35.1	77
24 hours	AC	4	6.34	5.7–7.7	41.27	36.3–45.1	47.61	42.4–52.8	87
	C57Bl	4	4.20	3.5–4.6	32.25	27.1–41.?	36.46	30.6–45.8	89

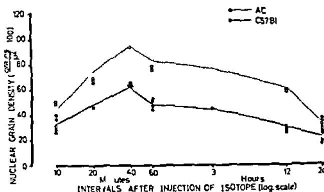


Fig 3

Mean values for nuclear grain density in the *zona glomerulosa* of AC and C57Bl mice at different intervals after injection of ^{3}H uridine The dots indicate the mean values for each animal The lines indicate the mean values for each experimental group

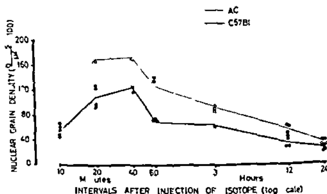


Fig 4

Mean values for nuclear grain density in the *zona fasciculata externa* of AC and C57Bl mice at different intervals after injection of ^{3}H uridine The dots indicate the mean values for each animal The lines indicate the mean values for each experimental group

Grain counts over the cytoplasm could be performed only in the *zona fasciculata externa* (p 4) In this zone the number of grains per cell increased in both strains during the first 20 or 40 minutes (Table 2) After 1 hour the values were somewhat lower Later the counts again increased and reached a maximum after 24 hours A previous study on the C57Bl mice (12) showed that ribonuclease treatment removed 92 per cent of the activity 20 minutes after the injection of isotope and 9[illegible] per cent when the animals were injected 24 hours before sacrifice These results indicate that both peaks of the incorporation curves represent RNA and not other radioactive substances but no satisfactory explanation of the temporary decrease after 1 hour can be given (For further discussion see reference 12)

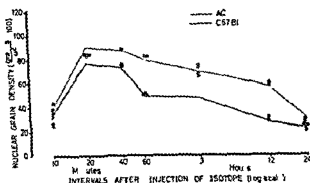


Fig 5

Mean values for nuclear grain density in the *ona fasciculata interna* of AC and C57Bl mice at different intervals after injection of ^3H uridine The dots indicate the mean values for each animal The lines indicate the mean values for each experimental group

Generally the incorporation of the RNA precursor into the nuclei was higher in the AC mice than in the control strain in all zones of the adrenal cortex (Figs 3–5) In the *zona glomerulosa* and *zona fasciculata externa* the mean values for nuclear grain density were higher than in the controls at all intervals except the 10 minute registration (Table 1) The mean values were higher in the AC group also at this interval but the single readings overlapped to some extent In the *zona fasciculata interna* the difference between the two strains was somewhat less than in the other zones but the mean values were higher in AC mice at all intervals

TABLE 3

Relative Values for Incorporation of ^3H Uridine into the 3 Adrenocortical Zones of AC and C57Bl Mice The Nuclear Grain Density (Grains/μ^3 × 100) of the Zona glomerulosa was given Unit Value in all Experiments Data from a Previous Study on Adrenocortical Stimulation and Inhibition are Included (Reference 12)

Experimental group	Zona glomerulosa	Zona fasciculata ext	Zona fasciculata int
	0 minutes labelling		
AC animals present series untreated	1 0	2 3	1 2
C57Bl animals present series untreated	1 0	2 3	1 6
C57Bl animals ACTH treated	1 0	3 4	3 3
C57Bl animals dexamethasone treated†	1 0	0 9	0 9
C57Bl animals hypophysectomized§	1 0	0 8	0 6
	60 minutes labelling		
AC animals present series, untreated	1 0	1 5	0 9
C57Bl animals present series untreated	1 0	1 4	1 0
C57Bl animals formalin stressed//	1 0	2 8	2 2

0 6 I u s c 3 times a day for 5 days
† 0 2 mg 2 times a day for 5 days
§ 41 59 hours survival
// 4 per cent solution 0 05 ml × 7 s c (4 hours interval)

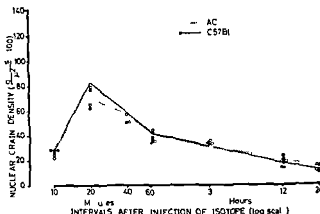


Fig 6

Mean values for nuclear grain density in the *medulla* of AC and C57Bl mice at different intervals after injection of ^{3}H uridine The dots indicate the mean values for each animal The lines indicate the mean values for each experimental group

In the *ona fasciculata externa* the number of grains in the cytoplasm was higher in the AC mice at all examined intervals (Table 2) The relative cytoplasmic labelling (number of grains over the cytoplasm in relationship to that of total cell) was similar in the two strains at all intervals

While the AC and C57Bl mice showed different labelling of the nuclei in all adrenocortical zones the *ratios* between the three zones were remarkably similar in the two strains (Table 3) This finding is important in view of previous investigations (12) which showed that these ratios changed when the adrenal cortex of C57Bl mice was stimulated and depressed (see Table 3)

The incorporation of precursor into the *medulla* was almost identical in the two strains (Fig 6 and Table 1) At one interval (20 minutes) the mean values for the groups differed somewhat but this was due to a very high labelling of one single C57Bl animal At the other intervals the differences between mean values are small and without any systematic trend

DISCUSSION

As has been discussed in some detail elsewhere (12) there is reason to believe that the autoradiographic method is a fairly sensitive indicator of the RNA metabolism in the adrenocortical cells The present study has demonstrated that the incorporation of ^{3}H uridine into the adrenal cortex was higher in AC males than in the control strain at all examined intervals The shape of the incorporation curves were similar in the two strains (Figs 3–5) and the strains also behaved similarly with regard to nuclear and cytoplasmic labelling (Table 2) Presumably these findings indicate that the adrenal cortex of the AC mice have a higher turnover of RNA per cell than normal ones A more

detailed interpretation is not possible on the basis of present observations

The finding of an active adrenocortical RNA metabolism in AC mice is consistent with the previous demonstration of a high amount of mitochondria in these cells (2) A previous histometric study on the nuclear and nucleolar cross sectional areas did not show any differences between the two strains (11) The method used in the present study is therefore probably a more sensitive indicator of the cellular RNA metabolism

The labelling of the adrenal *medulla* was nearly identical in the two strains (Fig 6) Thus there is no generalized difference in the cellular RNA metabolism in the strains This finding probably also indicates that the levels of precursor in the blood and tissues were of similar magnitude in the two strains

In a previous autoradiographic study the adrenal cortex was stimulated and depressed by changing the level of ACTH in various ways (12) In these experiments the incorporation of the isotope into the *ona fasciculata externa* and *interna* varied with the level of ACTH stimulation while the activity in the *zona glomerulosa* remained stable Variations in the level of ACTH thus caused changes in the ratios between the different zones In the AC males of the present study the incorporation was increased also in the *zona glomerulosa* and the ratios between the adrenocortical zones were similar in the two strains (Table 3) These findings suggest that the lipid depletion in the AC strain is not caused by an altered pituitary function but rather that the difference between the strains is inherent in the adrenal cortex itself

SUMMARY

The object of the present study was to investigate by autoradiography the incorporation of ^3H uridine into the adrenal cortex and medulla of mice with spontaneous adrenocortical lipid depletion (AC strain) and control mice (C57Bl strain) The adrenals were examined at intervals varying from 10 minutes to 24 hours after injection of the isotope

The incorporation into the adrenal cortex was higher in AC mice than in C57Bl animals at all intervals studied while the labelling of the medulla was nearly identical in the two strains The increased cortical labelling in AC mice was of roughly similar magnitude in all three adrenocortical zones These results were compared with a previous autoradiographic study on adrenocortical stimulation and inhibition in normal mice (12)

It is concluded that the adrenal cortex of mice with spontaneous adrenocortical lipid depletion probably has a higher turnover of RNA per cell than the control strain There is no indication that this difference is caused by an altered pituitary function

TABLE 1

Activities of Glucose 6 Phosphate Dehydrogenase and 6 Phosphogluconate Dehydrogenase in Adrenals of Normal (C57Bl strain) and Adrenocortical Lipid Depleted (AC strain) Mice Subjected to Different Nutritional Treatments

Nutritional treatment	Specific enzyme activities: Glucose 6 phosphate dehydrogenase			Specific enzyme activities: 6 phosphogluconate dehydrogenase			Per cent protein		
	C57Bl	AC	Difference	C57Bl	AC	Difference	C57Bl	AC	Difference
Stock diet	13.7	17.8	+4.1	3.57	4.84	+1.27	9.8	8.4	−1.4
Fasted	11.7	13.5	+1.8	5.1[illegible]	7.09	+1.97	11.4	12.0	+0.6
Carbohydrate diet	10.4	12.0	+1.6	4.43	6.[illegible]9	+1.8[illegible]	14.3	12.4	−1.9
Carbohydrate diet	10.6	16.4	+5.8	8.34	9.16	+0.82	8.3	8.1	−0.2
Carbohydrate diet	9.3	15.1	+5.8	8.26	10.08	+1.8[illegible]	9.8	7.8	−2.0
Mean	11.1	14.9	+3.8 ± 0.75 $p<0.005$	5.94	7.49	+1.55 ± 0.18 $p<0.001$	10.7	9.7	−1 ± 0.5 $p<0.1$

Nutritional treatments, tissue preparations and enzyme assay were as described in *Methods*. Enzyme activities are expressed as mμm NADPH formed per min per mg of protein (specific activity). Mean differences are presented together with the S.E. values.

TABLE 1

Activities of Glucose 6 Phosphate Dehydrogenase and 6 Phosphogluconate Dehydrogenase in Adrenals of Normal (C57Bl strain) and Adrenocortical Lipid Depleted (AC strain) Mice Subjected to Different Nutritional Treatments

Nutritional treatment	Specific enzyme activities: Glucose 6 phosphate dehydrogenase			6 phosphogluconate dehydrogenase			Per cent protein		
	C57Bl	AC	Difference	C57Bl	AC	Difference	C57Bl	AC	Difference
Stock diet	13.7	17.8	+4.1	3.57	4.84	+1.27	9.8	8.4	—1.4
Fasted	11.7	13.5	+1.8	5.12	7.09	+1.97	11.4	12.0	+0.6
Carbohydrate diet	10.4	12.0	+1.6	4.43	6.29	+1.88	14.3	12.4	—1.9
Carbohydrate diet	10.6	16.4	+5.8	8.34	9.16	+0.82	8.3	8.1	—0.2
Carbohydrate diet	9.3	15.1	+5.8	8.26	10.08	+1.82	9.8	7.8	—2.0
Mean	11.1	14.9	+3.8 ± 0.75 $p<0.005$	5.94	7.49	+1.55 ± 0.18 $p<0.001$	10.7	9.7	—1 ± 0.5 $p<0.1$

Nutritional treatments, tissue preparations and enzyme assay were as described in *Methods*. Enzyme activities are expressed as mμm NADPH formed per min per mg of protein (specific activity). Mean differences are presented together with the S.E. values.

6 *Molne K* The adrenal weight of mice with spontaneous adrenocortical lipid depletion A comparison with untreated ACTH and dexamethasone treated C57Bl mice Acta path microbiol scand 75 37–50 1969
7 *Molne K* *Histometric studies on the adrenal cortex of mice with spontaneous* adrenocortical lipid depletion A comparison with dexamethasone and ACTH treated C57Bl mice Acta path microbiol scand 75 51–63 1969
8 *Molne K* Gonadectomy and testosterone substitution in male mice with spontaneous adrenocortical lipid depletion A comparison with C57Bl mice Acta path microbiol scand 76 35–4? 1969
9 *Molne K* Autoradiographic studies on the incorporation of ^{3}H uridine into the adrenal glands of mice with spontaneous adrenocortical lipid depletion A comparison with C57Bl mice Acta path microbiol scand 77 353–362 1969
10 *Molbert E & Arnesen K* Elektronenmikroskopische Untersuchungen zur Ultrastruktur der Nebennierenrinde der weissen Maus Beitr path Anat. allg Path 122 31–56 1960
11 *Solem J H* The function of the adrenal cortex in mice with spontaneous adrenocortical lipid depletion Acta path microbiol scand 70 ?5–36 1967
12 *Stone D & Hechter O* Studies on ACTH action in perfused bovine adrenals The site of action of ACTH in corticosteroidogenesis Arch. Biochem *51* 457–469 1954
13 *Tepperman H M & Tepperman J* On the response of hepatic glucose 6 phosphate dehydrogenase activity to changes in diet composition and food intake pattern In Advances in Enzyme Regulation vol 1 121–136 Ed by G Weber Pergamon Press Oxford London New York Paris 1963
14 *Walters E & McLean P* Effect of thyroidectomy on pathways of glucose metabolism in lactating rat mammary gland Biochem J *105* 615 6?3 1967

Acta path microbiol scand 77 384-388 1969

The II Department of Pathology University of Helsinki Helsinki Finland.

MUCOSAL HYPERPLASIA IN THE GALLBLADDER DEMONSTRATED BY PLASTIC MODELS

By

G ELFVING H TEIR H DEGERT and V MÄKELÄ

Received 25 vi 69

Hyperplasia of the gallbladder mucosa even in cases without stones can be seen in histological sections and in surface photographs (1 3) This mucosal hyperplasia has been stated to be particularly typical in connection with cholesterosis (2)

The mucosa is thickened in hyperplastic cholecystoses of the gall bladder primarily for the reason that the villi have lengthened and often grown together (Fig 1)

With a view to obtaining a three dimensional picture of the changes of the inner surface of the gallbladder caused by mucosal hyperplasia the following method of reconstruction was elaborated by us

From a paraffin block prepared in the usual manner serial sections 40μ in thickness were made and stained Images of the sections were projected in proper succession on cardboard sheets on which the changes seen in each section were scrupulously traced Magnification in the projection was 50 fold The tracings on each sheet were transferred with equal care to a sheet of acrylic plastic from which the areas corresponding to air space were then cut out with the jigsaw As a result each plastic cut out constituted a faithful greatly enlarged replica of the respective original section Finally these replicas were stacked in proper succession and united by gluing whereby a reconstructed model of the gallbladder wall was obtained

Thirty successive specimens weer projected and reconstructed on plastic sheets 22 cm in length Their thickness was 2 mm corresponding exactly to the 50 fold magnification of 40μ The plastic model was thus 22 cm long and 6 cm deep which corresponds to a surface of 4 4 × 1 2 mm for a histologic specimen

MATERIAL

Reconstructions were made of (1) a normal gallbladder (2) gallbladder with primary mucosal hyperplasia (3 4) two gallbladders with cholesterolosis in addition and (5) a gallbladder presenting with emphysema Considering the great amount of work involved in the procedure study of a few typical cases had to suffice



Fig 1
Primary spongoid hyperplasia of the gallbladder mucosa × 50

RESULTS

The plastic reconstructions of the gallbladders under investigation were found to be highly useful and illustrative Mucosal hyperplasia of the gallbladder was far more distinctly evident in these than in common histological sections or surface photographs (Fig 2) of the same specimens

The mucosal folds are discrete and regularly arranged in the normal gallbladder (Fig 3) while in hyperplasia the formation of folds is disorganized The folds are deeper and they present greater diversity of shape (Fig 4) Removal of the portions corresponding to air spaces makes the funnel like and reticular formations which are so typical

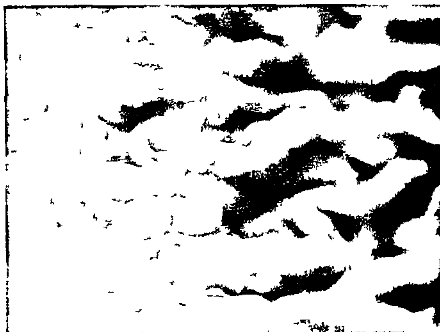


Fig 2
Surface photograph of the interior of a gallbladder with hyperplastic mucosa × 50

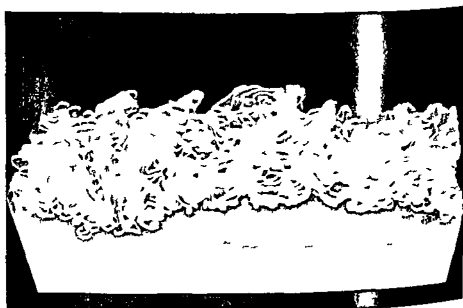


Fig 3
Plastic models of a normal gallbladder

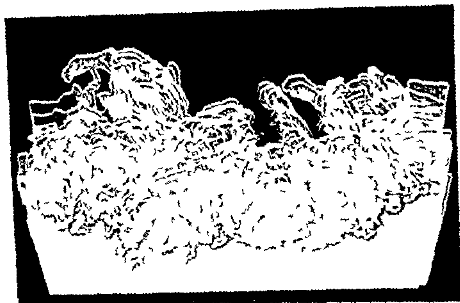


Fig 4
Plastic reconstruction of a case with hyperplasia of the mucosa

of the mucosa stand out in very clear relief The mucosa of the gall bladder is generally hyperplastic in cholesterolosis

CONCLUSIONS

The plastic models were clearly superior to ordinary histological sections which are two dimensional only The sample is also better illustrated by these than by ordinary surface photographs The reconstructions confirm the pronounced differences and dissimilarities which have been established in hyperplasia of the gallbladder mucosa At the same time they visualize with greater clarity the changes that can be seen in surface photographs and histological sections

The method is time consuming and difficult It follows that such reconstructions cannot be made in large series nor for any number of gallbladders However this will hardly be necessary since an accurate picture of the situation is obtained from the reconstructions and reliable illustration of the details of the mucosal changes has been achieved

SUMMARY

A method is described by which plastic reconstructions can be made of mucosal hyperplasia of the gallbladder for the purpose of comparing it with the condition of empyema and with the normal gallbladder The reconstructions made by the authors were perspicuous and they

served usefully in supplementing the picture furnished by ordinary surface photographs and histological sections

REFERENCES

1 *Elfving G Lehtonen T & Teir H* Clinical significance of primary hyperplasia of gallbladder mucosa Ann Surg *165* 61 1967
2 *Elfving G Palmu A & Teir H* Cholesterolosis and mucosal hyperplasia of gall bladder Ann Chir Gyn Fenn 57 28 1968
3 *Teir H Elfving G & Lehtonen T* Ramifying and adhesive mucosal folds of the gall bladder—A cause of distress? Acta path microbiol scand *66* 327 327

Acta path microbiol scand 77 389–398 1969

The University Institute for Experimental Medicine Norre alle 71
2100 Copenhagen O

EXPERIMENTALLY INDUCED CHANGES IN PLASMA ANGIOTENSINOGEN AND PLASMA RENIN

Studies on Normal Nephrectomized Ureterligated Hypoxic Partially Corticectomized or Medullectomized and Renal or Spontaneously Hypertensive Rats

By

Jens Bing and Knud Poulsen

Received 19 x 69

It is well known that nephrectomy causes a fall in plasma renin and an increase in plasma angiotensinogen (renin substrate) in many mammals (for literature see *Page & McCubbin* 1968) The decrease in plasma renin after nephrectomy is easily explained by the removal of the place of its formation and it is natural to explain the increased angiotensinogen concentration as secondary to the fall in plasma renin In order to elucidate the validity of this explanation plasma renin and angiotensinogen were determined not only in normal and nephrectomized animals but also in other conditions which either are known to or were supposed to influence renal function Partial corticectomy was performed in order to see if removal of most of the renin forming part of the kidneys would give the same results as nephrectomy removal of the non renin containing papilla by partial medullectomy served as a sort of control 24 hours ureterligated rats were studied because they have the same degree of uraemia as nephrectomized rats but no depletion of renal renin (*Gross et al* 1968) The renal and spontaneously hypertensive rats were included because of interest in the renin system in these conditions and the hypoxic rats because of the known influence of hypoxia on renal function

MATERIAL AND METHODS

Animals White female rats weighing 180–200 grams from the strain of Wistar (from Leo Pharm Trade Co were used for nearly all experiments The rats with spontaneous hypertension belonged to the strain of S A from the Robert Jones and Agnes Hunt Orthopaedic Hospital England.[1]

Partial *r e tomy* or *medullectomy* were performed as previously described

---

Supported by grants from *King Christian X s Foundation* and *The Danish Heart Association*

[1] The authors are thankfull to prof *Morten Simonsen* for supplying the S A rats

Acta path microbiol scand 77 389–398 1969

The University Institute for Experimental Medicine Nørre alle 71
2100 Copenhagen Ø

EXPERIMENTALLY INDUCED CHANGES IN PLASMA ANGIOTENSINOGEN AND PLASMA RENIN

Studies on Normal Nephrectomized, Ureterligated, Hypoxic, Partially Corticectomized or Medullectomized and Renal or Spontaneously Hypertensive Rats

By

JENS BING and KNUD POULSEN

Received 19 v 69

It is well known that nephrectomy causes a fall in plasma renin and an increase in plasma angiotensinogen (renin substrate) in many mammals (for literature se *Page & McCubbin*, 1968) The decrease in plasma renin after nephrectomy is easily explained by the removal of the place of its formation and it is natural to explain the increased angiotensinogen concentration as secondary to the fall in plasma renin In order to elucidate the validity of this explanation plasma renin and angiotensinogen were determined not only in normal and nephrectomized animals but also in other conditions which either are known to or were supposed to influence renal function Partial corticectomy was performed in order to see if removal of most of the renin forming part of the kidneys would give the same results as nephrectomy removal of the non renin containing papilla by partial medullectomy served as a sort of control 24 hours ureterligated rats were studied because they have the same degree of uraemia as nephrectomized rats but no depletion of renal renin (*Gross et al* 1968) The renal and spontaneously hypertensive rats were included because of interest in the renin system in these conditions and the hypoxic rats because of the known influence of hypoxia on renal function

MATERIAL AND METHODS

Animals White female rats weighing 180–200 grams from the strain of Wistar from Leo Pharm Trade Co were used for nearly all experiments The rats with spontaneous hypertension belonged to the strain of S A from the Robert Jones and Agnes Hunt Orthopaedic Hospital England[1]

Partial corticectomy or *medullectomy* were performed as previously describ d

Supported by grants from *King Christian X s Foundation* and *The Danish Heart Association*

[1] The authors are thankfull to prof *Morten Simonsen* for supplying the S A rats

(*Bing* 1949) After clamping of the renal artery *partial corticectomy* was performed by peeling off most of the cortical layer with a fine Graefe knife removing about 30 per cent of the total weight of the kidneys Thereafter the surface of the kidney was covered with a thin moist gelatine sponge (Spongostan) which after removal of the clamp was hold firmly around the operated kidney for a minute *Partial medullectomy* after clamping the renal artery an incision was made through the kidney whereafter the papilla was cut off with curved scissors as close to the rest of the medulla as possible Without use of gelatine sponge the two halves of the kidney were pressed together before and about one minute after removal of the clamp and fixed in this position with a silk thread *Partial clamping of the renal artery* was performed with silver clips as described by *Wilson & Byrom* (1939) *Hypoxia* was obtained by keeping the rats in a vacuum tank at a pressure of 300 mm Hg The air in the tank was renewed continuously and the pressure kept constant by help of a high vacuum pump (Speedivac) The changes from and to normal pressure in the tank was induced during about 15 minutes

The *concentration of angiotensinogen* in plasma was measured by the recently described method (1969) using the usual principle of incubating plasma with excess of renin after elimination of the angiotensinases and measuring the angiotensin formed by bioassay

In the experiments the results of which are given in Fig 3 the *angiotensinogen concentration* was determined by *radio immuno assay* the concentration of angiotensinogen S (expressed as nanograms angiotensin II per ml) is measured by a quantitative transformation to angiotensin II in an enzyme reaction with excess of renin and converting enzyme and abolished angiotensinase activity (*Poulsen* 1969) The angiotensin II is measured by radio immunoassay and expressed in terms of nanograms of angiotensin research Standard A

Renin concentration is measured as described (*Poulsen* 1969) The principle consists in a determination by radio immuno assay of the velocity of the decrease in the angiotensinogen concentration in the course of time The principle is characterized by a precisely determined concentration of angiotensinogen during the reaction controlled activity of the converting enzyme and complete abolishment of the influence of the angiotensinases The renin concentration (k F) is calculated from the equation

$$\ln \frac{S}{S - y} = (k \quad F) \quad t$$

(S_0) is the initial angiotensinogen concentration and (S − y) the angiotensinogen concentration at the time t The renin concentration (k F) is expressed in hours^{-1}

Renin activity = k F S_0 (ng ml^{-1} h^{-1}) is the angiotensin generation rate of the plasma (initial velocity) as calculated from the renin concentration (k F) and the initial angiotensinogen concentration (S) (*Poulsen* 1969)

The *haematocrit* determinations were performed by help of a Clay Adams autocrit and the plasma *creatinine* by help of the method of *Folin* and *Wu*

RESULTS

1 *Plasma Angiotensinogen Concentration*

The plasma angiotensinogen concentration was found to vary from 100 to 550 ng/ml in *normal rats* (Fig 1 A) with a mean of 325 ng/ml The effect of *nephrectomy* is seen in Fig 1 A which shows *the rise in the angiotensinogen concentrations with time* which are slightly increased already after 3 hours clearly increased 6 hours after nephrectomy steadily increasing to a maximum about 24 hours after the nephrectomy and then falling to somewhat lower but still clearly increased concentrations 48 hours after the operation While a similar

[2] The renin used was the standard donated by dr *Haas* to the W H O Lab for Biological Standards (Nat Inst Med Res M H Hill London) from where the angiotensin standard was also obtained

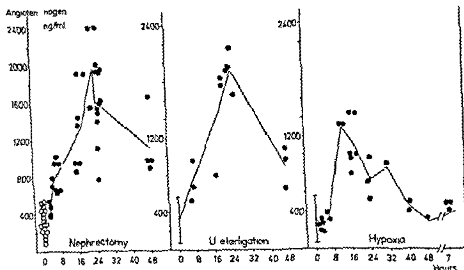


Fig 1

Fig 1 A B and C show the changes in angiotensinogen concentration (in ng angiotensin per ml) with time (in hours) after nephrectomy ureterligation and hypoxia respectively (●) In Fig 1 A the angiotensinogen concentrations of normal rats (○) are given and the range of these values are indicated in Fig 1 B and 1 C

curve is found for bilaterally *ureterligated rats* (Fig 1 B) the pattern for *hypoxic* rats kept at a pressure of 300 mm Hg (Fig 1 C) differs in the following ways the initial increase seems to be slower the maximum concentrations are reached already after 17 hours The concentrations are then falling to the upper part of the normal range which in the present study is seen to be reached about 40 hours after the start of hypoxia and still found in rats which were kept at the low pressure for 72 hours The Fig 2 B shows the maximal concentration reached in *hypoxic + nephrectomized* and in *hypoxic + ureterligated* rats in which there is a summation of the effects of hypoxia and nephrectomy or ureterligation on the angiotensinogen concentration the highest concentration being about 10 times the normal mean These results made it probable that the angiotensinogen concentrations in rats which were nephrectomized or ureterligated more than 40 hours after start of hypoxia would be identical with those found in non hypoxic nephrectomized or ureterligated rats This was also found to be true in four cases the concentrations of which (range 1100–2600) are not included in the Figures 24 hours after *partial corticectomy* or *partial medullectomy* the plasma angiotensinogen is markedly increased (Fig 2 C) the values in most cases being much higher than in nephrectomized or ureterligated rats and being about the same as those found in hypoxic + nephrectomized or hypoxic + ureterligated animals The two quite different operations (corticectomy and medullectomy) are seen to result in increase to the same high level of an

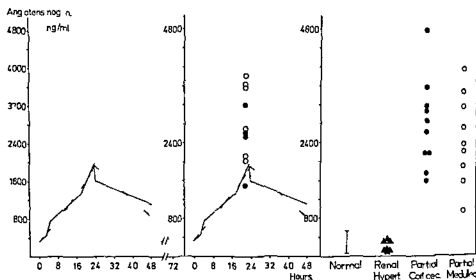


Fig 2

Fig 2 A shows lines through the mean values of angiotensinogen concentrations (in ng angiotensin per ml) of the nephrectomized (——) ureterligated (- - - -) and hypoxic ( ) rats shown in Fig 1 A B and C—Fig 2 B shows besides the lines given in Fig 2 A for nephrectomized (——) and ureterligated (- - - -) rats the summation of the effects of simultaneous hypoxia and either nephrectomy (○) or ureterligation (●)—Fig 2 C shows the range of the variations in angiotensinogen concentrations in normal rats and the concentrations found in renal hypertensive (▲) partially corticectomized (●) and partially medullectomized (○) rats

giotensinogen concentration In seven *renal hypertensive* rats which even when anaesthetized had blood pressures of 175 to 225 mm Hg and which had increased heart weights the plasma angiotensinogen concentrations were relatively low (Fig 2 C) with a mean of 190 ng/ml (range 69–344) while the mean of 7 simultaneously studied controls (which are not included in Fig 1) was 376 ng/ml (range 288–512) The angiotensinogen concentration in 8 rats with *spontaneous hypertension* was a little lower than that in normal rats the mean value being 265 ng/ml (range 207–369) as seen in Fig 3

2 *Plasma Renin Concentration and Activity*

In *normal* rats the *renin concentration* was found to vary from 0 010 to 0 040 h⁻¹ with a mean of 0 025 h⁻¹ (Fig 3) The figure further shows the well known extremely low concentrations found in 24 hours *nephrectomized* rats and it is seen that similar low concentrations are found in the partially *corticectomized* rats The concentrations both in *ureterligated* and *hypoxic* rats are about half the concentration found in normal rats the mean values being 40 and 60 per cent respectively of the normal mean similarly most of the partially *medullectomized* rats had concentrations as low as those found in hypoxic rats In *spontaneously hypertensive* rats the renin concentrations

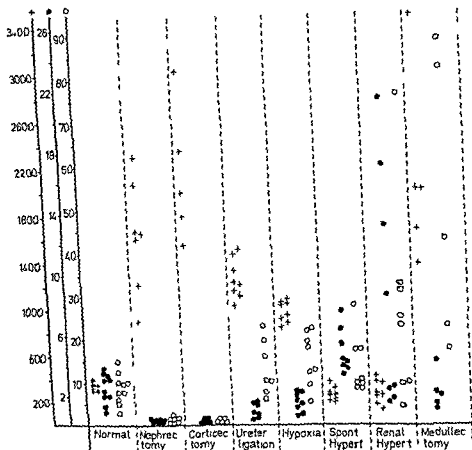


Fig 3

The relation between simultaneously determined *plasma angiotensinogen* (+) in ng angiotensin per ml *plasma renin concentration* (●) in hours⁻¹ 10 and *renin activity* (○) in ng ml⁻¹ hours⁻¹ The angiotensinogen values in this figure are *not* included in Fig 1 and 2

were markedly elevated and in *renal hypertensive* rats normal concentrations were found in 4 out of 8 animals while very high concentrations were found in the remaining 4 cases This division in two groups was not due to differences in duration of hypertension nor was there any correlation between renin concentration and blood pressure which in most cases was about 200 mm Hg

Because of the previously mentioned variations in the angiotensinogen concentration the changes in the *renin activity* do not simply follow the changes in renin concentration the only exception being the *nephrectomized* and partially *corticectomized* rats in which both concentration and activity are extremely low In the *ureterligated* and *hypoxic* rats the combination of low renin concentration and increased

angiotensinogen concentration results in normal or slightly increased activity. About the same renin activity is found in the *spontaneously hypertensive* rats because of the increased renin concentration and decreased angiotensinogen concentration. Markedly increased activity is found in most of the *renal hypertensive* and in all the partially *medullectomized* rats.

3. *Plasma Creatinine, Haematocrit, Blood Pressure and Body Weight*

While the hypoxic rats and the rats with spontaneous hypertension had normal plasma creatinine values of 0.8 to 1.2 mg per cent, normal or slightly increased values with a mean of 1.3 (range 0.3 to 1.6 mg%) were found in 10 renal hypertensive rats. Abnormally high values were found in all the operated animals, the values increasing with time after operation. In 24 hours nephrectomized or ureterligated rats the concentration was most often between 5 and 6 mg%, while somewhat lower values from 3 to 4 mg% were most often found in the partially corticectomized or medullectomized rats. The *haematocrit* values were nearly all in the normal range. *Blood pressure* did not change significantly from the normal mean of about 120 mm Hg in the nephrectomized, the ureterligated and the partially medullectomized rats, but the mean of the partially corticectomized rats was a little higher, 140 mm Hg with values from 120–180 mm Hg, while the blood pressure of the hypoxic rats was relatively low, from 60 to 93 mm Hg with a mean of 82 mm Hg. The blood pressure of the spontaneously hypertensive rats was about 200 mm Hg. The *body weight* was rather unchanged in all but the hypoxic rats who lost in weight when kept at low pressure for more than 48 hours although they had access to their normal food and water.

DISCUSSION

1. *Plasma Angiotensinogen*

The values for plasma angiotensinogen concentration in *normal* and *nephrectomized rats are close to those reported in the literature (Carretero & Gross (1967), Page & McCubbin (1968))* including previous studies by the author (1969). The present study contains three different materials from normal rats: the first (17 rats) given in Fig. 1A, the mean of which is 325 ng, the second and smaller (7 rats) with a mean of 376 collected as a direct control to the concentrations found in renal hypertensive rats, and the third normal material (10 rats) with a mean of 351 consisting of the determinations shown in Fig. 3. The mean angiotensinogen concentration in all 34 normal rats is thus 350 ng. The reason for the high degree of scatter of the values, especially found in the first material (Fig. 1A), is probably due to the fact that these concentrations were found in studies performed on materials during a rather long period of time (some months), while the other normal materials were collected during a shorter space of time (about a week).

The fact that the scatter of the values for the normal angiotensinogen concentration is relatively small compared with the enormous changes found in the various groups of rats studied under pathological conditions makes it probable that there is a regulation of the angioten

sinogen concentration in normal rats but that this regulation is broken under various pathological conditions In nephrectomized rats the rather uniform increase in the concentration per hour during the first 24 hours (Fig 1 A and 2 A) makes it probable that the increase is due to lack of the normal enzymatic splitting of angiotensinogen by renin According to this explanation the increase of 2000–350 = 1650 ng/ml/24 hours would mean that the normal angiotensinogen formation should be about 60 ng/ml/hour This value however does not conform with the renin activity of about 10 ng/ml/hour obtained in the present study (Fig 3) Another observation which does not agree with the proposed explanation of the cause of the increase in angiotensinogen concentration in nephrectomized rats is the close parallelism between the changes in the concentrations with time in nephrectomized and ureterligated rats (Fig 1 A and B and 2 A) although the decreases in plasma renin concentration differ markedly (Fig 3)

The resemblance between the curves in nephrectomized and ureterligated rats (Fig 2 A) makes it reasonable to try to find a common cause of the changes The equal degree of uraemia could be such a common cause and uraemia could also be part of the cause of the very high angiotensinogen concentrations found in the partially corticectomized and in the partially medullectomized rats (Fig 2 C) If the uraemic state is able to cause an increase in plasma angiotensinogen concentration it is not the only cause of such a change This is seen from the finding that angiotensinogen concentrations in many corticectomized or medullectomized rats are higher than concentrations in nephrectomised or ureterligated although the plasma creatinine values were a little higher in the last two groups The conclusion that uraemia cannot in any case be the only cause of increase in angiotensinogen concentration is confirmed by the finding of high angiotensinogen concentrations in the non uraemic hypoxic rats (Fig 1 C and 2 A) The different causes of the increase in angiotensinogen in nephrectomized or ureterligated rats and in hypoxic rats are made probable by the different curves seen in Fig 2 A and is further seen in Fig 2 B which shows the summation of the effect of simultaneous nephrectomy or ureterligation and hypoxia

The normal or low angiotensinogen concentrations found in the *renal hypertensive* rats contrast with the increased concentrations found by *Carretero* and *Gross* (1967) the cause of the difference between these results being unknown

2 *Plasma Renin*

The very low renin concentration found in nephrectomized rats is identical with that found by many previous investigators and is easily explainable by loss of the renin forming kidneys The similarly low values found in partially (sub totally) corticectomized rats are equally

natural as the renin formation is known to take place in the epitheloid cells of the juxtaglomerular apparatus most of which are removed by the operation Less easily explainable is the about halving of the renin concentration both in the (non uraemic) hypoxic rats and in the (uraemic) ureterligated which finding contrasts with the increased renin secretion found by *Vander* and *Miller* (1964) 10 to 60 min after ureteral occlusion The renin concentration in the spontaneously hypertensive rats of the S A strain is higher than that found in our normal Wistar rats The highly varying values found in the renal hypertensive rats are in accordance with findings by previous investigators As mentioned above the very high values could not be correlated to either duration of degree of hypertension nor to plasma creatinine concentration but there was in most cases lower body weight higher relative heart weight and more pronounced hypertensive vascular disease in these rats which were also more weak than the hypertensive animals with normal plasma renin concentration

3 *Relation between Plasma Renin and Angiotensinogen*

Previous investigators have rendered it probable that angiotensin exerts a negative feedback control of the renin release (*Vander & Geelhoed* (1964) *Genest et al* (1966) and *Klaus & Heizmann* (1967)) at the same time showing that other factors such as the state of sodium balance and aldosterone secretion play a role for the magnitude of the plasma renin activity

Experimentally induced alterations in the plasma angiotensinogen concentration could lead to a change in the angiotensin generation rate of the plasma and assuming the existence of the negative feedback mentioned above exert a change in the renin secretion rate In accordance with this an inverse correlation between renin activity and angiotensinogen concentration in plasma or the renin content of the kidneys and the plasma angiotensinogen concentration has been found under some conditions but this has in no way been a constant finding (*Aida* (1963) *Carretero & Gross* (1967))

In the present study there is in most groups a marked inverse correlation between plasma renin concentrations and plasma angiotensinogen concentrations When the values from the individual rats were compared correlation between plasma renin and angiotensinogen concentration was however not found This means that the present findings agree with the idea that plasma angiotensinogen concentrations possibly through the plasma angiotensin concentration exerts a negative feedback control of the renin release from the kidneys but that other factors play a decisive role

SUMMARY

The concentrations of plasma angiotensinogen and renin were markedly changed after experimentally induced changes in renal morphology and function. A mutual regulation of the angiotensinogen concentration and the renin concentration was only probable in so far as most of the groups of differently pretreated rats showed an inverse correlation between plasma angiotensinogen and plasma renin while this was not a regular finding when the values from the individual rats were compared.

The increases in angiotensinogen concentration with time were so similar after nephrectomy and ureterligation that a common cause of stimulation is probable. Depletion of plasma renin seems not to be the cause as the plasma renin concentration was much lower in nephrectomized than in ureterligated rats. As both nephrectomized and ureterligated rats had the same degree of uraemia the uraemic state could be the cause of the increase in plasma angiotensinogen. The finding of still higher angiotensinogen concentrations in partially corticectomized or partially medullectomized rats indicate however that uraemia in these cases can at least only be partly responsible for the increase in plasma angiotensinogen.

Increase in plasma angiotensinogen was further found to be provoked by hypoxia. That the stimulations were due to different causes in the uraemic nephrectomized and in the non uraemic hypoxic rats was shown by differences in the increase in plasma angiotensinogen with time in nephrectomized (or ureterligated) and hypoxic rats and by a summation of the effects on the angiotensinogen concentration in hypoxic + nephrectomized (or hypoxic + ureterligated) rats.

Plasma renin concentration was extremely low in nephrectomized and partially corticectomised rats, about half the normal in ureterligated hypoxic and most of the partially medullectomised rats, elevated in rats with spontaneous hypertension and normal or markedly elevated in renal hypertensive animals.

REFERENCES

Aida M, Maebashi M, Yoshinaga K & Ishinohe F: Renin and Renin substrate in Man. Tohuko J exp Med 87 35–39 1965.

Bing J: Effects of partial corticectomy and partial medullectomy on the kidneys of various mammals. Acta Med Scand Suppl 234 26–32 1949.

Bing J: Reinvestigation of the cause of the increased velocity of reaction between renin and angiotensinogen in plasma from nephrectomised rats. Acta path microbiol scand 75 33–36 1969.

Carretero O & Gross F: Evidence for humoral factors participating in the renin substrate reaction. Circ Res 20/21 Suppl II 115–126 1967.

Carretero O & Gross F: Renin substrate in plasma in the rat. Am J Phys 213 695–700 1967.

Genest J, de Champlain J, Veyrat R, Boucher R: Factors controlling renin in man. Internat Club on Arterial Hypertension Paris 49–54 1966.

Gross F Carretero O & Johnston C I Effect of Ureteral Ligation on Blood Pressure and Renin Content of the Kidneys in Renal Hypertensive Rats Proc of Soc Exp Biol Med *128* 935–940 1968
Klaus D & Heinmann A Zur Regulation der Reninsekretion durch Angiotensin Klin Woch *45* 657–659 1967
Page I H & McCubbin J W Renal Hypertension Chicago 1968
Poulsen K Estimation of plasma renin concentration using radioimmunoassay of angiotensin II Scand J Clin Lab Invest in press 1969
Vander A J & Geelhoed G W Inhibition of Renin Secretion by Angiotensin II Proc Soc Exp Biol Med *120* 399–403 1965
Vander A J & Miller R Control of Renin Secretion in the anaesthetized dog Am J Phys *207* 537–546 1964
Wilson C & Byrom F B Renal changes in hypertension Lancet i 136–141 1939
Hirasawa K et al In recently published papers increase of renin substrate was found in ureterligated Jap Circ J *32* 1591–1595 1968
Gould A B & Goodman S A In hypoxic rats Abstracts I IV Int Congr Nephrol Stockholm page 200 1969
Skinner S L et al The hypothesis that a feedback normally operates tending to maintain plasma renin activity constant as substrate levels alter was further discussed in studies Clin Sci *36* 67–76 1969
Rosset E & Veyrat R Acta endocr Suppl *138* 122 1969
Menard J & Millie P C R Acad Sc Paris *268* 1749 1752 and 2710–2713 1969

NOTE ADDED IN PROOF

In recently published papers increase of renin substrate was found in ureterligated (*K Hirasawa et al* Jap Circ J *32* 1591–1595 1968) and in hypoxic rats (*A B Gould & S A Goodman* Abstracts I IV Int Congr Nephrol Stockholm page 200 1969) The hypothesis that a feedback normally operates tending to maintain plasma renin activity constant as substrate levels alter was further discussed in studies of *S L Skinner et al* (Clin Sci *36* 67–76 1969) *E Rosset & R Veyrat* (Acta endocr Suppl *138* 122 1969) and *J Menard & P Millie* (C R Acad Sc Paris *268* 1749 1752 and 2710–2713 1969)

Acta path microbiol scand 77 399 404 1969

The Second Department of Pathology University of Helsinki the Fourth Medical Clinic Helsinki University Central Hospital and Kivela Hospital Helsinki

A CYTOCHEMICAL METHOD FOR THE STUDY OF BILE CANALICULI IN FINE NEEDLE ASPIRATES OF THE LIVER

By

CURT WASASTJERNA

Received 12 v 69

Although diagnostic fine needle puncture of the liver is an easy and safe procedure the information obtained is rather limited in scope if the cytological specimens are stained by standard methods such as May Grunwald Giemsa or Papanicolaou Tumour cells are detectable in a high percentage of cases with cancer metastases (*Soderstrom* 1966 *Lundquist* 1968 a) and the cytological diagnosis of hepatitis is practicable in many cases (*Lundquist* 1968 b) but the diagnostic value of histological specimens is usually superior for other purposes However histological biopsy with punch needles is not without risk (*Sherlock* 1968) Accordingly different staining procedures should be tried to derive more information from cytological specimens The enzymatic activity of the liver cells is high and valuable results can thus be expected from enzyme cytochemical methods

The bile canaliculi are some of the most interesting structures of the liver Their function is important under normal circumstances and some forms of liver disease result from changes in the canaliculi (*Schaffner & Popper* 1959 *Popper* 1968) For the most part the canaliculi have been studied by electron microscopy (*Popper* 1967) For light microscopy the canaliculi can be visualized by staining frozen sections for alkaline phosphatase (*Gomori* 1941 *Wachstein* 1959 *Bitensky* 1967) or for ATPase (*Wachstein & Meisel* 1957 *Novikoff et al* 1958 *Wachstein* 1959) The latter method provides better results but is rarely applied in routine histology for clinical purposes

Great value would be attached to a simple staining method for the evaluation of bile canaliculi in cytological specimens Among a number of cytochemical procedures tested staining for the demonstration of an amino acid naphthylamidase was found to give the best results

---

Aided by a grant from the *Sigrid Juselius Foundation* A part of the clinical material was very kindly supplied by the staff of the Aurora Hospital and of the Second and Fourth Surgical Departments of the Helsinki University Central Hospital

METHOD

Cytological liver specimens were obtained from hospital patients with the instrument designed by *Franzén* in 1955 (*Franzén et al* 1960). The outer diameter of the needle is 0.7 mm. Large livers were often punctured by the abdominal approach. In other cases the site of puncture was usually the 9th intercostal space in the mid axillary line. A small drop of tissue fluid was drawn into the needle and smeared on glass slides by the conventional haematological technique. The smears were allowed to dry at room temperature. In addition to special stainings, one slide was always stained by the May Grunwald Giemsa method.

Staining for the demonstration of amino acid naphthylamidase was effected by a modification of the procedure described for haematological purposes by *Rutenberg & Rosales* (1966). The smears were fixed in a cold (about 4 C) mixture of equal parts of chloroform and acetone for 3 minutes, rinsed with running water and dried. They were incubated in the following solution for 1 hour at 37 C.

Acetate buffer 0.1 M pH 6.5	25 ml
Sodium chloride solution 0.85 per cent	20 ml
Substrate stock solution	2.5 ml
KCN solution 0.02 M	2.5 ml
Fast Garnet GBC	25 mg

The substrate stock solution was prepared by dissolving 80 mg of alanyl-4-methoxy-2-naphthylamide (Cyclo Chem Corp.) in 10 ml of distilled water. After incubation the smears were rinsed in distilled water for 2 minutes and counterstained with Haemalum for 5 minutes. The smears were then rinsed with tap water, dried and covered with glycerol gel and a cover slip.

RESULTS

The positive reaction attained by application of this method is a brilliant red colour. Normal liver cells were either negative or displayed a slight granular reaction, usually concentrated to the periphery of the cell. The colour of the cytoplasm was light yellow both after the ordinary staining procedure and in controls incubated without substrate. No red colour was seen in the controls. The enzyme activity was somewhat diminished by the fixation used. If the smears were fixed for shorter periods or without chloroform the intracellular activity was more marked but the background staining was more pronounced and the contrast poorer.

The amino acid naphthylamidase of the liver canaliculi seems to be rather stable. In most cases the smears were fixed a few hours after the aspiration and stored for some days at + 4 C until stained, however comparable results were obtained with unfixed smears stored at room temperature for up to 5 days. The colours of the stained smears did not change in about 6 weeks but a slight distortion of the pattern was discernible as a result of shrinkage of the glycerol gel used for mounting the cover slips. All the other mounting media tested as well as immersion oil on uncovered smears have been found to dissolve the stain almost immediately. Most of the enzyme activity was concentrated in the bile canaliculi. These were distinctly visualized as about 1.5 μm wide bright red bands forming networks between the cells (Figs 1, 2). The canaliculus border was sharp. Usually a black crystallized structure was visible in some parts of the preparations mostly



Figs 1–4
Fig 1 Normal bile canaliculi × 250 Fig 2 Normal canaliculi × 1250 Fig 3 Infectious hepatitis × 500 Fig 4 Obstructive jaundice × 500

in thicker tissue fragments but the canaliculi were never disturbingly obscured. No black precipitation occurred if Garnet GBC was replaced by fast blue B salt in the incubation solution. The canaliculi were distinctly visualized with the latter coupling agent but the contrast was not as good as with Garnet GBC and details of the canaliculus wall were not as well distinguishable. Therefore the author prefers Garnet GBC. Some smears were stained for the demonstration of alkaline phosphatase by an azo coupling method (*Merker & Heilmeyer* 1960). The canaliculi were not visualized by this procedure.

Eighty aspirates have been studied. The results in liver disease will be reported in detail when a more extensive material has been compiled and analysed and after the completion of some animal experiments. However, the first impression is a wide variability of canaliculus pattern in different liver disorders. In mild cases of virus hepatitis the walls have seemed to be irregular or broken. In more severe cases the continuity of the canaliculus system was lost and in the most severe cases only irregular red patches were observable around the periphery of liver cells (Fig. 3). In the few cases of cirrhosis studied to date the granular intracellular enzyme activity has been increased. The appearance of the canaliculi varied between the normal pattern and distorted bands. In some areas no canaliculi were seen and other cell groups seemed to contain an irregular proliferation of short canaliculi. In extrahepatic obstructive jaundice the canaliculi were dilated and their borders more diffuse than is normally the case (Fig. 4). On occasion the red colour covered parts of the surrounding cells but as a rule the continuity of the network was preserved.

DISCUSSION

The fine needle aspiration of liver cells is safe and almost painless to the patient. The staining method described here is fast and simple and suitable for routine use. The bile canaliculi are stained as distinct red bands and can thus be studied in great detail. They seem to be very resistant. Sometimes the adjoining cells have been torn off but the canaliculi are still intact.

The bile canaliculi are specialized parts of the liver cell membranes (*Popper* 1968) and seem to contain a much higher activity of amino acid naphthylamidase than other parts of the cells. No effort has been made to find out whether the enzyme activity is specific for a particular amino acid. This is probably not the case but the substrate now used is evidently highly suitable for morphological studies. In normal liver the staining of the canaliculi was remarkably uniform and exhibited a distinct pattern on microscopic study at high magnification. It may thus be concluded that the enzyme activity is probably mainly a characteristic of the canaliculus wall and not at least exclusively produced by bile in the canaliculi. If fresh human bile obtained from

r drains of cholecystectomized patients was smeared on albumin covered glass slides and these were stained by the present method only a faint pink colour was distinguishable

The negative results of staining for alkaline phosphatase indicate that this enzyme is rinsed away during the fixation and staining procedure It is probably mainly a component of the bile In histological specimens of normal dog livers *Aronsen et al* (1968) found alkaline phosphatase activity in the epithelium of the ductules and in the peri portal canaliculi and sinusoids Liquid tissue components are better preserved in histological specimens than in thin smears but small intra and extracellular details are often better distinguishable in smears

The morphology of the canaliculus network is disturbed in liver disease In most cases so far studied the pathological pattern could be easily distinguished from the normal morphology However the pattern varied considerably from case to case in similar disorders occasionally even from one cell group to another in the same smear Accordingly a morphological diagnosis was not always possible Never theless the study of the canaliculus morphology contributed to the final diagnosis in many cases More experience is required but it can already be stated that the method provides valuable information about the canaliculi and it may thus be a useful diagnostic procedure for the examination of patients with liver disorders

SUMMARY

A simple enzyme staining method for visualization of the bile cana liculi in cytological liver specimens is described Alanyl 4 methoxy 2 naphthylamide is used as substrate and Garnet GBC as coupling agent In normal liver the canaliculi are seen as regular red bands against a light yellow background In liver disease the canaliculus network is disturbed and several different patterns are encountered in different diseases The intracellular activity varies between negative and a mo derate granular reaction

REFERENCES

1 *Aronsen K F Hagerstrand I & Norden J G* Enzyme studies in dogs with extra hepatic biliary obstruction Scand J Gastroenterol 3 355–358 1968
2 *Bitensky L* Histochemistry of liver disease In *Read* The liver Butterworth Lond n 1967 p 61 63
3 *Franzén S Giertz G & Zajicek J* Cytological diagnosis of prostatic tumours by transrectal aspiration biopsy a preliminary report Brit J Urol 32 193–196 1960
4 *Gomori G* The distribution of phosphatase in normal organs and tissues J cell comp Physiol *17* 71–83 1941
5 *Lundquist A* Snabbdiagnostik av levermetastaser XXX Medicinska Riksstämman Stockholm 1968 a p 193–195
6 *Lundquist A* Cytologiska synpunkter på hepatitmaterial Nord Med 80 1414 1968 b

7 *Merker H & Heilmeyer L* Die alkalische Phosphatase neutrophiler Leukozyten Dtsch med Wschr *85* 253–258 1960
8 *Novikoff A B Hausman D H & Podber E* The localization of adenosine triphosphatase in liver in situ staining and cell fractionation studies J Histochem Cytochem *6* 61–71 1958
9 *Popper H* Electron microscopy in the diagnosis of liver disease In Read The liver Butterworth London 1967 p 47–53
10 *Popper H* Cholestasis Ann Rev Med *19* 39–56 1968
11 *Rutenberg A M & Rosales C L* Histochemical method for the demonstration of leukocyte naphthylamidase using two substrates alanyl and methionyl 4 methoxy 2 naphthylamide Brit J Haemat *12* 595–597 1966
12 *Schaffner F & Popper H* Morphologic studies of cholestasis Gastroenterology *37* 565–573 1959
13 *Sherlock S* Diseases of the liver and biliary system Blackwell Oxford 1968
14 *Soderstrom N* Fine needle aspiration biopsy Almqvist & Wiksell Stockholm 1966
15 *Wachstein M* Enzymatic histochemistry of the liver Gastroenterology *37* 525–537 1959
16 *Wachstein M & Meisel E* Histochemistry of hepatic phosphatase at a physiologic pH Amer J clin Path *27* 13–23 1957

Acta path microbiol scand 77 405-413 1969

The Department of Pathology College of Veterinary Medicine
Helsinki Finland

THE ULTRASTRUCTURE OF BETA CELL ISLET TUMOUR IN A DOG

By

Timo Rahko

Received 8 iv 69

Staining methods used in the identification of normal alpha and beta islet cells do not always produce clearly defined tinctorial reactions when applied to islet cell tumours of man (*Frantz* 1959 *Greider & Elliot* 1964 *Georgsson & Wessel* 1967 *Toker* 1967a) or dog (*Wilkinson* 1964 *Marcus et al* 1964) At ultrastructural level however the neoplastic alpha and beta cells of man can be differentiated without histochemical techniques (*Lacy* 1959 *Lazarus & Volk* 1962 *Bencosme et al* 1963 *Greider & Elliott*, *Toker* 1967 a b)

Islet cell tumours of animals verified with light microscopy are infrequently encountered in the available literature The cases reported previous to the papers of *Bullock* (1965) and *Rouse & Wilson* (1966) are included in the review of literature by *Roulatt* (1967) Altogether twenty four insulomas of dog are on record so far This electron microscopic study reveals that beta cell islet tumour of dog can be identified by the ultrastructure of cytoplasmic granules in the tumour cells

CASE HISTORY AND METHODS

A ten year old Finnish Harrier bitch was subject to attacks of exhaustion under exercise Clinical examination *revealed* a mammary tumour and lesions in the heart and pancreas were suspected Without more detailed examinations the dog was euthanatized and an immediate autopsy carried out A whitish fairly firm spherical tumour 6-8 mm in diameter was noted superficially in the middle of the duodenal portion of the *pancreas* The macroscopic and microscopic investigations revealed a non metastasizing adenocarcinoma in the mammary gland in addition to the pancreatic tumour

For light microscopy formalin fixed tissue from the pancreatic tumour non neoplastic pancreas and from the *pancreas of a healthy* dog were embedded in paraffin cut at 5 μ and stained with Gomori s (1939) aldehyde fuchsin and chrome haematoxylin phloxine by *Hansen* s (1949) method for beta granules with *Launoy* s method for zymogen granules and with haematoxylin eosin (H & E) Van Gieson and periodic acid Schiff (PAS)

Requests for reprints should be addressed to Dr *Timo Rahko* Department of Pathology College of Veterinary Medicine II meentie 57 *Helsinki* 55 (Finland)

This investigation was supported by a grant from *L ak tehdas Orion Scientific Research Foundation*

For electron microscopy small blocks of tissue of the pancreatic tumour and of the non neoplastic pancreas were fixed at 4 C in 2 5 per cent glutaraldehyde in 0 1 M phosphate buffer for 2 hours washed in the same buffer plus 10 per cent sucrose and postfixed at 4 C in 1 per cent osmium tetroxide in the same buffer for 2 hours. All tissues were rapidly dehydrated in a graded series of ethanol solutions and embedded in Epon (*Kay* 1965) Sections cut at 1 μ were stained with 1 per cent basic fuchsin in 50 per cent acetone for light microscopic identification of tissue in blocks (*Winkelstein et al* 1963) Thin sections were cut with glass knives using an *Ultro tome* ultramicrotome mounted on copper grids stained with aqueous solutions of uranyl acetate (*Kay*) and examined with an *Akashi* electron microscope

RESULTS

Light Microscopy

The encapsulated tumour presented an abundant stroma consisting of anastomotic bands of fibrous tissue and thin walled blood capillaries The parenchyma was composed of irregular islets and cords of cells In an area of the tumour cell groups resembling acinar and tubular structures were noted (Fig 1) A homogenic PAS positive substance filled the lumen of some tubular looking structures Immediately around this tissue appearing exocrine the neoplastic islet cells were pleomorphic presenting an irregular nucleus and a scanty often finely vacuolated cytoplasm Mitotic figures in these cells were fairly frequent The exocrine looking tissues inside the tumour seemed to originate from neoplastic islet cells instead of being entrapped by the expanding tumour

The greatest part of the tumour consisted cords of predominantly rectangular cells with a small spherical or elongated nucleus The cytoplasm was abundant faintly eosinophilic and finely granular (Fig 2) With specific stains only a few tumour cells presented a profusion of beta granules like normal beta cells in islets surrounding the tumour Moreover occasional beta granules could be demonstrated in some tumour cells but most cells were devoid of them

---

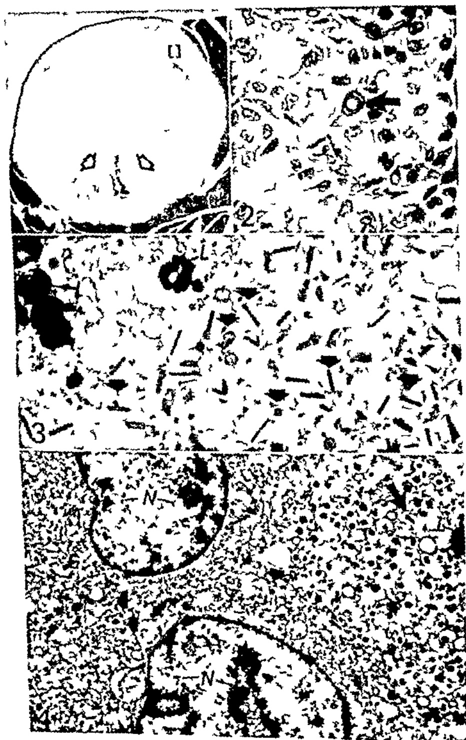
Figs 1 4

Fig 1 Low power photomicrograph showing the capsule with adjacent pancreatic parenchyma and general topography of beta cell islet tumour The arrows point to an area with dark stained structures resembling exocrine acini The square area in the adjoining section of the tumour is shown in detail in Fig 2 H & E × 13

Fig 2 High power photomicrograph showing a cluster of well differentiated rectangular neoplastic islet cells Inclusion like structure with granular inner matrix encircled by membrane in the nucleus of a tumour cell (arrow) Van Gieson × 540

Fig 3 Cytoplasm of beta cell from *non neoplastic* islet presenting numerous beta granules (*e g* arrows) Intrasaccular rectangular bar shaped or spherical cores of granules surrounded by clear space L lipid body × 14 000

Fig 4 Sections of three adjacent tumour cells In the cell to the right a cluster of beta granules with predominantly spherical core separated by clear space from surrounding smooth membrane Bar shaped beta granule (long arrow) In cells to the left a few spherical beta granules (short arrows) N nucleus G Golgi zone L lipid body × 11 000


2
3
N
N

A structure resembling an inclusion and consisting of a finely granular eosinophilic matrix occurred in many nuclei (Fig 2) The roundish structure enclosed in a membrane was located in the nucleoplasm sometimes in contact with the nuclear membrane Similar structures occurred in the nuclei of some non neoplastic islet cells No other significant changes were noted in the pancreatic parenchyma

Electron Microscopy

The different cells in the non neoplastic pancreatic islets were identified according to the descriptions of ultrastructure in the relevant literature (*Lacy* 1957 1961 *Herman et al* 1964 *Munger et al* 1965 *Sato et al* 1966) The beta cells presented numerous cytoplasmic granules consisting of an electron opaque central core separated by a translucent halo from the limiting agranular membrane of a spherical profile The profile of the central core was rectangular bar shaped or sometimes roundish (Fig 3)

A varying number of cytoplasmic granules of the same size as the normal beta granules occurred in the tumour cells (Fig 4) The roundish or bar shaped cores were surrounded by a broad translucent space and limited by a smooth membrane of a spherical profile (Fig 5) Most granules were similar to the beta granules in the non neoplastic beta cells The relative number of spherical beta granules however was greater in the tumour cells than in the non neoplastic beta cells (Fig 6)

Most cells presented only few granules an abundance of membraneous structures and unattached ribosomes Rough endoplasmic reticulum formed parallel profiles and dilated cisternae and occasionally separate spherical structures (Figs 5 and 7) Cisternae and membraneous structures of smooth endoplasmic reticulum were distributed at random in the cytoplasm Aggregates of cisternae with a smooth surface sometimes resembled the Golgi complex (Fig 4) The size and structure of mitochondria was not uniform (Fig 8) A few lipid bodies occurred in the cytoplasm (Fig 4)

Chromatin had a tendency to marginalization (Fig 9) Nuclei in

---

Figs 5–7

Fig 5 Tumour cell p r in beta granule (left) Spherical beta granule (long arr w) Part of parallel array f granular endoplasmic reticulum (ER) in area adjoining nucleus (N) NL nuclear envelope CM cell membrane Numer us beta granules in the cell t the right Bar shaped b ta granules (short arr s s) × 18 000

Fig 6 Tum ur cell with man spherical and a few bar shaped (arrows) beta granules N nucleus Nl nucle lu CH chromatin × 21 000

Fig 7 Tum ur cell p or in beta granules Spherical beta granule (arr w) An abundant r ugh end plasmic reticulum (ER) f rming parallel arrays cisternae and spherical tructures N nucleus M mitochondria × 21 000


GER
CM
NE
N
CH
N
NU
GER
M
N
7

A structure resembling an inclusion and consisting of a finely granular eosinophilic matrix occurred in many nuclei (Fig 2) The roundish structure enclosed in a membrane was located in the nucleoplasm sometimes in contact with the nuclear membrane Similar structures occurred in the nuclei of some non neoplastic islet cells No other significant changes were noted in the pancreatic parenchyma

Electron Microscopy

The different cells in the non neoplastic pancreatic islets were identified according to the descriptions of ultrastructure in the relevant literature (*Lacy* 1957 1961 *Herman et al* 1964 *Munger et al* 1965 *Sato et al* 1966) The beta cells presented numerous cytoplasmic granules consisting of an electron opaque central core separated by a translucent halo from the limiting agranular membrane of a spherical profile The profile of the central core was rectangular bar shaped or sometimes roundish (Fig 3)

A varying number of cytoplasmic granules of the same size as the normal beta granules occurred in the tumour cells (Fig 4) The roundish or bar shaped cores were surrounded by a broad translucent space and limited by a smooth membrane of a spherical profile (Fig 5) Most granules were similar to the beta granules in the non neoplastic beta cells The relative number of spherical beta granules however was greater in the tumour cells than in the non neoplastic beta cells (Fig 6)

Most cells presented only few granules an abundance of membraneous structures and unattached ribosomes Rough endoplasmic reticulum formed parallel profiles and dilated cisternae and occasionally separate spherical structures (Figs 5 and 7) Cisternae and membraneous structures of smooth endoplasmic reticulum were distributed at random in the cytoplasm Aggregates of cisternae with a smooth surface sometimes resembled the Golgi complex (Fig 4) The size and structure of mitochondria was not uniform (Fig 8) A few lipid bodies occurred in the cytoplasm (Fig 4)

Chromatin had a tendency to marginalization (Fig 9) Nuclei in

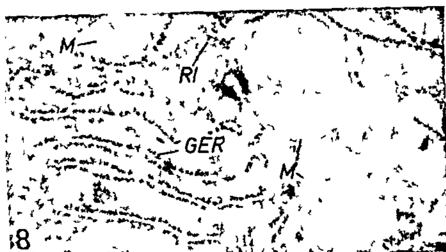
---

Figs 5–7

Fig 5 Tumour cell poor in beta granules (left) Spherical beta granule (long arrow) Part of parallel arrays of granular endoplasmic reticulum (GER) in area adjoining nucleus (N) NM nuclear envelope CM cell membrane Numerous beta granules in the cell to the right Bar shaped beta granules (short arrows) × 18 000

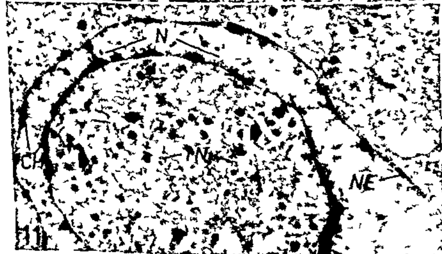
Fig 6 Tumour cell with many spherical and a few bar shaped (arrows) beta granules N nucleus NU nucleolus CH chromatin × 21 000

Fig 7 Tumour cell poor in beta granules Spherical beta granule (arrow) An abundant rough endoplasmic reticulum (GER) forming parallel arrays cisternae and spherical structure N nucleus M mitochondria × 21 000


M
RI
GER
M
8


GER
9
N
CH


N
10


N
CH
Nu
NE
11

the invading fraction of cytoplasm is only apparently separate and does not form a true intranuclear structure

SUMMARY

A light and electron microscopic investigation of a beta cell islet tumour in a ten year old Finnish Harrier is reported

The encapsulated tumour had a general morphology characteristic of an endocrine neoplasm of relatively low malignancy With specific stains beta granules could be demonstrated in a few tumour cells

Tumour cells presented a regular ultrastructure and an abundance of cytoplasmic organelles Cytoplasmic granules of the same ultrastructure as normal beta granules occurred in small numbers Spherical beta granules dominated in the tumour cells whilst rectangular beta granules were frequent in the examined non neoplastic beta cells

REFERENCES

Bencosme S A Allen R A & Latta H Functioning pancreatic islet cell tumors studied electron microscopically Amer J Path 42 1–21 1963

Bullock I Two cases of a functioning islet cell tumor in the canine Calif Vet 19 14–17 30–31 1965

Frantz V K Tumors of the Pancreas Atlas of Tumor Pathology Fascicle 27 Armed Forces Institute of Pathology Washington D C 1959

Georgsson G & Wessel W Vergleichende elektronmikroskopische Untersuchungen normaler menschlicher Pankreasinseln und eines hormonellaktiven *Inselzell* Carcinoms mit Hyperinsulinismus Z Krebsforsch 69 70–86 1967

Gomori G A differential stain for cell types in the pancreatic islets Amer J Path 15 497–499 1939

Greider M H & Elliott D W Electron microscopy of human pancreatic tumors of islet cell origin Amer J Path 44 663–678 1964

Hansen H J Insulom hos hund Vet Med 1 363–370 1949

Herman L Sato T & Fitzgerald P J The pancreas In Kurtz Stanley M (Ed) *Electron Microscopic Anatomy Academic Press New York and London* 1964 p 59 65

Kay D H Techniques for Electron Microscopy 2nd ed Blackwell Oxford 1965

Lacy P E Electron microscopic identification of different cell types in the islets of Langerhans of the guinea pig rat rabbit and dog Anat Rec *128* 255 267 1957

Lacy P E Electron microscopic and fluorescent antibody studies on islets of Langerhans Exp Cell Res Suppl 7 296–308 1959

Lacy P E Electron microscopy of the beta cell of the pancreas *Amer J Med 31* 851 8 9 1961

Lazarus S S & Volk B W Histochemical and electron microscopic studies of a functioning insulinoma Lab Invest *11* 1279 1291 1962

Marcus F C Bucci T J & Kraner K L Pancreatic islet cell tumor in a dog J Amer vet med Ass *145* 1198 1203 1964

Munger B L Caramia F & Lacy P E The ultrastructural basis for the identification of cell types in the pancreatic islets II Rabbit dog and opossum Z Zellforsch 67 776 798 1965

O Shea J D Islet cell intranuclear inclusions in the canine pancreas Res vet Sci 5 138 142 1964

Rouse B T & Wilson M R A case of hypoglycaemia in a dog associated with neoplasia of the pancreas Vet Rec 79 454–456 1966

Rowlatt U Spontaneous epithelial tumours of the pancreas of mammals Brit J Cancer *21* 8 107 1967

T Herman L & Fit gerald P J The comparative ultrastructure of the pan creatic islet of Langerhans Gen Comp Endocrinol 7 132–157 1966
:r C Observations on the ultrastructure of a metastasizing beta cell islet tumor Diabetes *16* 800–803 1967 a
:r C Some observations on the ultrastructure of a malignant islet cell tumor associated with duodenal ulceration and severe diarrhea J Ultrastruct Res *19* 52?–531 1967 b
\insson J S Clinico pathologic conference School of Veterinary Medicine University of Pennsylvania J Amer vet med Ass *144* 404–412 1964
'kelstein J Menefee M G & Bell A Basic fuchsin as a stain for osmium fixed Epon embedded tissue Stain Techn *38* 20?–?04 1963

TABLE 2

Results of Oral Inoculation into Mice of Serial Dilutions of a Suspension Containing 50 000 Cysts/ml. Dosage 0.2 ml/Mouse

Inoc. dilut.	No. of mice inoc.	No. of mice dead/inoc.	No. of mice pos./inoc. Dye test	No. of mice pos./inoc. Brain cysts	No. of pass. mice pos./inoc. Dye test	No. of pass. mice pos./inoc. Brain cysts
Undil.	6	6/6	–	–	24/24	24/24
1:10	6	1/6	5/6	5/6	4/4	4/4
1:10²	6	0/6	6/6	6/6	–	–
1:10³	6	0/6	4/6	4/6	–	–
1:10⁴	6	0/6	0/6	0/6	–	–
1:10⁵	6	0/6	0/6	0/6	–	–
1:10⁶	6	0/6	0/6	0/6	–	–
1:10⁷	6	0/6	0/6	0/6	–	–

inoculation. Blood was obtained from four; two had a titre of 1:10 (9th day) and two had 1:50 (11th day) in the dye test. Six mice received 1 000 cysts and one of these died 10 days after inoculation. All surviving mice were bled and examined in the dye test 18 days after inoculation. The remaining five of six mice that received 1 000 cysts each were positive with titres of 1:250, and so were six mice that received 100 cysts. Six mice were given 10 cysts each and four became positive; two had a titre of 1:250 and two had 1:50. The mice in the remaining groups were all negative, including six mice that received a dosage equivalent to one cyst per mouse. The passage mice were positive, most of them with a titre of 1:1250, a few with 1:250 in the dye test. Numerous *Toxoplasma* tissue cysts were seen in the brains of all serologically positive mice and none were observed in the negative mice. All control mice were negative.

This experiment was repeated but this time the mice were ino-

TABLE 3

Results of Intraperitoneal Inoculation of Serial Dilutions of a Suspension Containing 50 000 Cysts/ml. Dosage 0.2 ml/Mouse

Inoculum dilution	No. of mice inoculated	No. of mice dead/inoc.*	No. of mice pos./inoc. Dye test	No. of mice pos./inoc. Brain cysts
Undiluted	6	0/6	6/6	6/6
1:10	6	0/6	6/6	6/6
1:10²	6	2/6	4/6	4/6
1:10³	6	0/6	5/6	5/6
1:10⁴	6	0/6	1/6	1/6
1:10⁵	6	0/6	0/6	0/6

* Two mice died 18 and 19 days after inoculation respectively but were not examined.

ulated intraperitoneally Results are presented in Table 3 The mice were bled and examined 22 days after inoculation Six mice that received 10 000 cysts were positive with titres of 1 250 in the dye test and so were six mice that received 1 000 cysts each Of the six mice that were inoculated with 100 cysts each two died 18 and 19 days after inoculation respectively These two mice were not examined but the remaining four were positive Six mice received 10 cysts each and five became positive In the group that was inoculated with a calculated dosage of one cyst per mouse one out of six became positive with a titre of 1 250 in the dye test All the mice in the last group remained negative and so did all control mice *Toxoplasma* tissue cysts were demonstrated in the brains of all serologically positive mice whereas none were seen in the dye test negative mice

Single cysts were micro isolated and inoculated intraperitoneally into each of four mice Another four mice were used as controls These mice had been pre bled and examined in the dye test, and were negative with a titre of less than 1 5 They were bled and examined again 18 days after inoculation and all four were positive with titres of 1 250 Numerous *Toxoplasma* tissue cysts were found in their brains The control mice had remained negative

When the four cats in this group were again fed infected mice about three months later and faecal separations were prepared as before it did not result in any cyst formation On microscopical examination of the faecal material no cystic structures were seen nor were any nematode ova observed All the mice inoculated with faecal material remained serologically and parasitologically negative

Group II

Microscopical examination Cysts as described above were found in the faeces of all three cats in this group From cat 6 they were seen in the faeces collected during the second week after feeding while from cats 7 and 8 they were found in the material from both the first and second week Besides the cysts numerous bacteria and fungi were seen But no other protozoan cysts were observed nor were any nematode ova seen

Mouse inoculation The results obtained in this experiment are very similar to the results described in group I (Table 4) Mice inoculated with material containing cysts were dying within 5–13 days The longest time of survival was noted for mice fed material collected during the first week from cat 8 Four out of six mice in that group died after 11–13 days while the remaining two recovered and survived to autopsy after six weeks One mouse out of six fed material from the first week of cat 6 became serologically and parasitologically positive although no cysts were noted in the material on microscopy

A total of 23 passage mice from cat 6 63 from cat 7 and 40 from

TABLE 4

Results of Feeding Mice Faecal Material from 3 Experimentally Toxoplasma Infected Nematode Negative Cats Dosage 1 ml Per Mouse

Cat n	Weekly periods	Cysts	Mortality of mice		Surviving mice		Passage mice
			Dead/inoc	Surviv time	DT pos/inoc	Brain cysts	Pos/inoc
6	Control	–	0/6		0/6	0/6	
	1	—	0/6		1/6	1/6	
	2	+	6/6	6–10 days		–	23†/24
	3	—	0/6		0/6	0/6	
7	Control	—	0/6		0/6	0/6	
	1	+	6/6	6–7 days	–	–	19/24§ 20/20
	2	+	6/6	5–9 days	–	–	24/24
	3	–	0/6		0/6	0/6	
8	Control	—	0/6		0/6	0/6	
	1	+	4/6	11–13 days	2/6	2/6	16/16
	2	+	6/6	6 9 days	–	–	24/24
	3	—	0/6		0/6	0/6	

In faecal material

† One mouse died within 24 hours after inoculation and was not examined

§ Five mice died within 1[illegible]–15 days after inoculation The brain of each was inoculated into four mice

cat 8 were examined in the dye test They were all positive with titres from 1 1250–1 6250 On microscopy of their brains numerous *Toxoplasma* tissue cysts were seen An equal number of control mice were negative

Serological examination of the cats was done at the beginning and at the end of the experiment All three cats had become positive with a rise in dye test titre from less than 1 5 to 1 250

DISCUSSION

Group I

The idea of a possible connection between the cystic organisms described above and *Toxoplasma* infection arose on the basis of the microscopical findings compared with the results of mouse inoculation The cysts were present in fairly large numbers in the material obtained during the first and second period and the mice inoculated with this material died in six or seven days Eight out of these 12 mice had developed *Toxoplasma* antibodies although in low titres before dying The third group however inoculated with cyst free material remained negative This seemed to point to the cyst as causative agent Apparently there was a correlation between the presence of cysts in the inoculum the clinical illness or death of the mice and the development of *Toxoplasma* antibodies

In order to give further support to the idea of such a correlation the additional experiments were performed Inoculation into mice of serial dilutions of a counted suspension was done twice Oral and intraperitoneal inoculation were used *Sum & Hutchison* (1966) had demonstrated that intraperitoneal inoculation of faecal material from cats could result in *Toxoplasma* infection in mice It is noteworthy that in neither of these experiments did any mouse become positive when inoculated with a dilution representing less than one cyst per mouse On the other hand only one mouse out of twelve became positive when inoculated with a calculated dosage of one cyst However when dealing with so few organisms there are several incidental factors which will influence the outcome Some of the cysts may not be viable A single cyst may adhere to either syringe or needle and not get into the mouse It may also in the case of oral inoculation pass through the intestinal canal without excysting and without causing any infection The one mouse in this experiment which became positive after receiving one cyst was inoculated intraperitoneally When comparing the two routes of infection it is also remarkable that 10 000 cysts are sufficient to kill a mouse when administered orally but not intraperitoneally We cannot give any definite explanation of this but it seems likely that it is due to differences in the process of excystation In the intestinal canal under the influence of the various digestive juices all the organisms may be released within

a short interval of time In the peritoneal cavity the release may be more gradual enabling the mouse to survive the acute stage of the infection

The technique of micro isolation is more suitable for handling single organisms than is the above method The amount of suspending medium is kept at a minimum and the micropipette used for isolation is also used for the subsequent inoculation into the mouse To prevent any possible loss through the intestinal canal the four single cysts were inoculated intraperitoneally and atypical cysts or cysts that seemed to be damaged or non viable were avoided

Removal of all cysts from a suspension of faecal material and inoculation into mice of material cleared of cysts in order to demonstrate the removal of toxoplasmic infectivity with the cysts has been considered repeatedly However for technical reasons we have decided not to attempt such an experiment In some of our suspensions we have seen that bursting of cysts may occur outside of a host Since we do not know how many parasites this would release and what size they would be we feel that neither micro isolation nor filtration would ensure a complete removal of all cystic elements

All results seem to indicate that the cystic organism is the infective agent in these experiments Two alternative possibilities to this are 1) the cyst demonstrated in faeces is not responsible for the infection in mice 2) the infection observed in the mice is not caused by *Toxoplasma gondii*

Arguments against the first proposition are that firstly only bacteria and fungi were observed in the inoculum and no protozoan cysts other than those described were seen Secondly no other living organisms were apparent in the same concentration as the cyst if any other organisms were responsible for the infection this would have to be the case Thirdly *Hutchison et al* (1969) have carried out similar infection experiments in SPF cats in which identical cysts were demonstrated in the faeces

Arguments against the second proposition are that it is highly unlikely that any foreign organism would be capable of forming antibodies and tissue cysts in the brains of mice indistinguishable from those demonstrable in *Toxoplasma* infection

Group II

This experiment was performed in order to reproduce the results in nematode negative and *Toxoplasma* negative cats Unfortunately the cats were not examined post mortem for the presence of nematodes However the faecal material was examined on many occasions by different investigators and nematode ova were never seen Moreover all the cats were given Piperazine Adipate prior to the experiment because this was found to be more effective than Vermiplex So

it seems that in this experiment cyst formation and transmission of *Toxoplasma* were independent of nematodes

Furthermore it was demonstrated that feeding whole mouse carcasses is not necessary to initiate cyst formation Cat 6 was fed infected mouse brains only This means that the influence of any possible cystic forms present in the mouse intestinal epithelium can be excluded

The formation site of the cystic form is unknown However the fact that the cysts in these experiments were produced and shed within 4 5 days seems to indicate that formation might take place in the alimentary tract Studies to investigate this problem are being performed but have not yet been completed

The serological status of the cat prior to the experiment and the way this influences cyst formation is rather interesting and —from an epidemiological point of view—important The presence of dye test antibodies in the cat indicating previous exposure to the parasite does not necessarily interfere with cyst formation On the other hand it can also from the above experiments be concluded that every *Toxoplasma* infected meal will not be followed by cyst formation It looks as if a certain interval of time between exposures was necessary

We are convinced that we are dealing with a new form of *Toxoplasma gondii*, but further studies will be required to fit it into the life cycle of the parasite We are aware of the fact that this cyst is remarkably similar to the isosporan oocyst However we are not prepared to discuss its relationships until more detailed information is available

SUMMARY

A cystic organism exists in the faeces of cats experimentally infected with *Toxoplasma* it seems capable of producing *Toxoplasma* antibodies and *Toxoplasma* tissue cysts in mice An association between this cystic organism and infections in mice by injection of serially diluted inocula and by micro isolations has been demonstrated

REFERENCES

Aajaard K Laboratory methods for the diagnosis of congenital toxoplasmosis In J Chr Siim (editor) Human toxoplasmosis Munksgaard Copenhagen 1960 p 206–210

Dubey J P Studies on *Toxocara cati* larvae infected with *Toxoplasma gondii* J Protozool 14 suppl p 42 1967

Dubey J P Isolation of *Toxoplasma gondii* from the faeces of a helminth free cat J Protozool *15* 773–775 1968

Frenkel J K Dubey J P & Miller N L *Toxoplasma gondii* Fecal Forms Separated from Eggs of the Nematode *Toxo ara cati* Science *164* 432–433 1969

Hutchison W M Experimental transmission of *Toxoplasma gondii* Nature *206* 961–962 1965

Hutchison W M The nematode transmission of *Toxoplasma gondii* Trans R Soc Trop med Hyg *61* 80–89 1967

Hutchison W M Dunachie J F & Work K The faecal transmission of *Toxoplasma gondii* Acta path microbiol scand *74* 462–464 1968

Hutchison W M, Dunachie J F & Work K Unpublished results 1969

Sabin A B & Feldman H A Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoan parasite (*Toxoplasma*) Science *108* 660–663 1948

Sheffield H G & Melton M L *Toxoplasma gondii* Transmission through Faeces in Absence of *Toxocara cati* Eggs Science *164* 431–432 1969

Siim J Chr & Hutchison W M Unpublished results 1966

Work K & Hutchison W M A new cystic form of *Toxoplasma gondii* Acta path. microbiol scand *7*, 191–192 1969

Acta path microbiol scand 77 425–432 1969

Institute of Medical Microbiology University of Aarhus Aarhus C Denmark

THE POSSIBLE ROLE OF CIRCULATING INTERFERON ON AUTOINTERFERENCE IN MICE INFECTED INTRAPERITONEALLY WITH WEST NILE VIRUS

By

SVEN HAAHR

Received 4 vii 69

Autointerference expresses itself as protection or reduced viral multiplication in hosts inoculated with large doses of virus which in smaller doses is pathogenic and multiplies to high levels (19)

Autointerference in vivo has been observed in connection with intracerebral titration in mice with louping ill (8) yellow fever (23) dengue fever (18 21) and Rift Valley fever (14) viruses and with yellow fever virus inoculated subcutaneously in galagos (22) The phenomenon has probably been observed in several other laboratories without being reported

Attempts have been made to explain the phenomenon as either resulting from the formation of an incomplete virus which might have a blocking effect on suceptible cells (14) or as the consequence of an infection caused by a mixture of non modified and modified virus particles (18) The possible role of interferon on autointerference in vivo does not appear to have been studied

As we in this laboratory have a strain of West Nile virus producing autointerference on intraperitoneal inoculation in mice we have undertaken a study designed to determine what role interferon might play in this phenomenon

MATERIALS AND METHODS

Most materials and methods have been described in detail in previous papers (10 11)

Viruses *West Nile virus* strain Egypt 101 in its 4th mouse brain passage in this laboratory was used for the infectivity experiments This strain was originally obtained from Casals in 1961 Stocks of West Nile virus were prepared by intracerebral inoculation of virus into suckling mice On the development of symptoms of encephalitis usually after 4 days the brains were removed after preceding exsanguination and ground with sand in 10 parts of PBS (pH 7 3) After centrifugation at 6000 rpm for 30 minute volumes of 1 ml of the supernatant were stored at −70 C

Semliki forest virus (SF strain Smithburn in its 6th mouse brain passage in this laboratory was used as challenge virus in titration of interferon. This strain was originally obtained from Casals in 1961 The virus stock was prepared as described above under WN

Animals Male and female albino mice of a non inbred strain were used In most

of the experiments the animals weighed 23-26 gm but as given in the text some experiments were made with mice of other weights Eight or ten mice were used in each experimental group

Processing of specimens Removal of blood was done as described previously (10) The pooled blood was centrifuged at 3000 rpm for 10 minutes The serum was pipetted off and used for virus titration and for interferon assay Serum dilutions used for interferon assay were dialysed against Sørensen s buffer pH 2 and after 48 hours at 4 C dialysed back to pH 7 4 After centrifugation at 3000 rpm for 30 minutes the supernatant was used for the assay

Interferon assay was performed as previously described (10) by the plaque inhibition method in secondary cultures of mouse embryonic cells Interferon titres expressed as units per 100 μl of serum were recorded as the reciprocal of the highest dilution which reduced by 50 per cent the number of plaques (SF) counted in the controls i e 50 per cent plaque-depressing dose (PDD_{50}/100 μl)

A stock reference preparation of interferon at known titre was used in each assay to detect any changes in the sensitivity of the system

Virus titration The virus content of the serum was determined by intracerebral inoculation into mice aged 2–4 days of 10 fold serial dilutions in PBS The virus titre was expressed as the exponent of the logarithmic ($\log_{10}$) dilution per 20 μl of serum which caused death in 50 per cent of the animals as calculated by the method of *Karber* (12)

The virus doses given in the experiments are determined by intracerebral titration in mice aged 2–4 days

Haemagglutination inhibition test (HI) was carried out using the standard techniques described by Clark and Casals (4) Sera were tested against two HA units beginning at a 1 10 dilution

Neutralization test Sera diluted 1 2 1 10 and 1 50 in PBS were mixed with five doses of LD_{50} West Nile virus After standing at 37 C for 60 minutes the mixture was inoculated intracerebrally in 2–4 day old mice

Steroid Hydrocortisate (Hydrocortisoni acetas NFN Løvens kemiske Fabrik Copenhagen) suspended in water was administered subcutaneously near the tail root in doses of 10 mg per mouse 4 hours prior to inoculation with West Nile virus Control mice were inoculated in the same way with equivalent doses of PBS

Characteristics of viral inhibitor The viral inhibitor found in the serum dilution exhibited the characteristics described in a previous paper (10) The properties are in keeping with mouse interferon as described by other investigators (5)

RESULTS

Autointerference at Different Ages

The autointerference phenomenon was found to be closely correlated to the age and weight of the mice Results obtained in mice of varying

TABLE 1

Mortality in Mice of Varying Age and Weight after Intraperitoneal Inoculation of West Nile Virus in Doses of 10^6 LD_{50} and 10^3 LD_{50}

Weight and age	10^6 LD_{50} No dead/no tested	10^6 LD_{50} Mortality %	10^3 LD_{50} No dead/no tested	10^3 LD_{50} Mortality %
2 gm/2–4 days	10/10	100 %	10/10	100 %
17–19 gm/c 3 weeks	13/16	81 %	16/16	100 %
23–26 gm/c 5 weeks	18/40	45 %	35/40	88 %
30–32 gm/c 7 weeks	2/16	13 %	7/16	44 %

age and weight are given in Table 1 As is shown the phenomenon was not seen in mice weighing 2 gm (2 4 days old) In 17 19 gm mice (3 weeks of age) there was 100 per cent mortality with the lowest virus dose and approximately a 20 per cent reduction in mortality when the highest virus dose was given In older mice presenting a higher resistance against all virus doses an even higher degree of autointerference was found

Virus and Interferon in Serum during the First 24 Hours Post inoculation

A study was then performed to determine whether there was any difference in viral and interferon content in the serum during the first 24 hours after intraperitoneal inoculation in groups of mice receiving 10^6 and 10^3 LD$_{50}$ doses respectively of West Nile virus In Table 2 the results of a typical experiment are given

As shown in the table interferon could not be demonstrated in the serum at any point of time within the first 24 hours in the group of mice receiving 10^3 LD$_{50}$ and virus could first be demonstrated in the serum after 24 hours

In the group where 10^6 doses of West Nile virus was given interferon could be demonstrated in the serum after six hours Thereafter increasing amounts of interferon were found during the next 12 hours a maximum being reached after 18 hours After 18 hours the amount of interferon decreased Virus could be demonstrated two hours after virus injection but not after six hours After 12 hours virus could again be demonstrated and thereafter in increasing amounts

TABLE 2

Virus and Interferon Content of Pooled Serum from 8 Mice during the First 24 Hours after Intraperitoneal Inoculation of West Nile Virus in Doses of 10^6 LD and 10^3 LD$_{50}$

Hours after virusinfect	10^6 LD$_{50}$ Virus	10^6 LD$_{50}$ Interferon	10^3 LD$_{50}$ Virus	10^3 LD$_{50}$ Interferon
2	1 5	<4§	<0	<4
6	<0	10		
12	0 35	32	<0	<4
18	2 1	128		
24	1 35	32	1 15	<4

Log LD$_{50}$/20 µl
§ PDD$_{50}$/100 µl

Virus and Interferon in Serum During the First Week Post inoculation

In other experiments viral and interferon content in the serum was investigated during the following days in the two groups Fig 1 shows

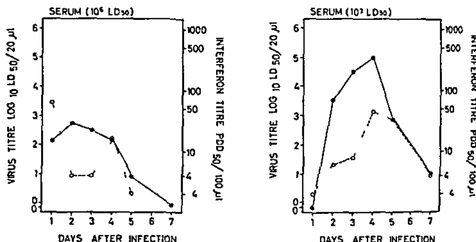


Fig 1

Virus (●——●) and Interferon (O--O) content of pooled serum from 10 mice after intraperitoneal inoculation of West Nile virus in doses of 10^6 LD_{50} and 10^3 LD_{50}

the results obtained in one of these experiments In the group receiving 10^3 LD_{50} doses of virus the content of interferon and virus was in accordance with previously performed studies (10) i e, increasing through the fourth day and thereafter decreasing In the experiments presented here the viraemia maximum was particularly high Usually maximum occurred during the fourth day with a titre of about 10^4 LD_{50} In the group receiving 10^6 LD_{50} large amounts of interferon could be demonstrated 24 hours after virus injection which is in agreement with the above but during the following days slight or no virus inhibiting activity could be found in the serum Viral content remained at approximately the same level through the fourth day and was consistently lower than in the groups receiving a smaller virus dose In all studies performed a less pronounced viraemia was found from the second day in the groups of mice receiving the larger virus dose

Virus and Interferon in Serum on Autointerference at Different Ages

The relationship between the age of the mice and the appearance of autointerference prompted a comparison of interferon induction and viral content in the serum in mice of various ages in which autointerference is seen Table 3 shows the findings from such a study where intraperitoneal injections of 10^6 LD_{50} doses of West Nile virus were given to groups of mice weighing approximately 17-19 gm and 25-27 gm As will be seen a higher viral and interferon content was constantly found in the younger mice where autointerference was less pronounced

TABLE 3

Virus and Interferon Content of Pooled Serum from Groups of 8 Mice of Different Ages after Intraperitoneal Inoculation of West Nile Virus in Doses of 10^6 LD_{50}

Hours after virus inoculation	Mice weighing 17–19 gm		Mice weighing 2[illegible]–[illegible]7 gm	
	Virus	Interferon	Virus	Interferon
24	1 7	32	0 9	16
48	[illegible] 5	16	1 5	4
72	2 3	<4	<1	<4
96	1 5	<4	0 7	<4

Absence of Autointerference in Steroid Treated Mice

Previous studies (11) have demonstrated inhibition of interferon induction in mice given steroids prior to virus inoculation Autointerference was therefore studied in steroid treated mice An inoculum corresponding to 10^4 LD_{50} doses of virus was given intraperitoneally in untreated mice and mice treated with 10 mg hydrocortisate 4 hours prior to virus inoculation The untreated mice showed autointerference whereas mortality was 100 per cent in the steroid treated mice Viral and interferon content in serum from two similar groups of mice was studied during the course of infection and is shown in Table 4 As expected a reduction in interferon induction was found in the steroid treated mice Viral content in the serum was found to be almost the same in the two groups during the first 24 hours but hereafter a markedly higher titre was seen in the steroid treated mice

TABLE 4

Virus and Interferon Content of Pooled Serum from 8 Mice Each Mouse being Treated with 10 mg of Hydrocortisate or a Corresponding Volume of Saline 4 Hours before Intraperitoneal Inoculation of West Nile Virus in Doses of 10^6 LD_5

Hours after virus inoculation	Saline		Hydrocortisate	
	Virus	Interferon	Virus	Interferon
2	2 1	<4	1 9	<4
6	<1	12	<1	<4
18	[illegible] 3	64	2 1	16
48	1 3	<4	3 0	≦4
72	<1	<4	3 3	<4
1[illegible]0	1 1	4	3 1	<4
144	<1	<4	[illegible] 7	<4

Effect of Various Virus Doses in Immune Response

Antibody production in the two groups of mice was elucidated by studying haemagglutinating and neutralizing antibodies responses in individual mice in the two groups Results as regards HI antibodies

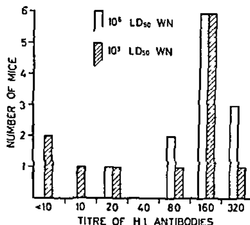


Fig 2

Number of mice with different titres of HI antibody 8 days after inoculation West Nile virus in doses of 10^6 LD50 and 10^3 LD50

eight days after virus inoculation are given in Fig 2 There was significant difference ($p > 0.10$ rank sum test) in the production HI antibodies in the two groups Neutralizing antibodies could not demonstrated eight days after virus inoculation

DISCUSSION

A detailed explanation as to why autointerference appeared on intr peritoneal inoculation with the strain of West Nile virus used he cannot be given on the basis of the present study Among the facto considered to be of importance for the host resistance to viral infectic (2) only antibody and interferon production were examined

As regards the role of antibody no significant difference was foun in the development of haemagglutination inhibiting antibodies in th two groups of mice receiving different doses of virus Neutralizin antibodies were not found in the serum eight days after viral injectio at a time when virus in West Nile infections in mice normally ha invaded the central nervous system (10) Antibody production there fore could not have had any decisive influence in reducing mortalit in the mice given the larger virus inoculum This is in accordance wit the minor role usually attributed to antibody production in the defenc mechanism against primary viral infection (2)

In the group where autointerference was observed the interfero content in the serum was found to be relatively high at a time whe actual viraemia had not yet developed (the virus demonstrated in th serum two hours after viral injection probably resulted from spillove from the inoculated virus) As protection against neurotropic vir infections in mice often has been demonstrated when interferon has been injected or induced shortly before or after virus injection (3 6

9 16 20) it appears that this early circulating interferon could ave played a decisive role in the reduced mortality found in these ice

Suggestive evidence of direct protection against a virus which had timulated interferon induction as found in the present experiments loes not appear to have been obtained previously in *in vivo* experi nents

The lack of autointerference demonstrated after steroid treatment urther supports the supposition that early interferon induction is of mportance for the development of autointerference as early inter feron induction was inhibited in these mice These findings do not however provide an unequivocal answer as steroid treatment is supposed to inhibit cellular immunity (17) also thought to be of im portance for the resistance to primary viral infection (1)

It is difficult to reach definite conclusions concerning the increased autointerference seen in older mice The amount of circulating inter feron and virus was lower in older mice findings that are in agreement with other studies (13 15) Generally the maximum level of viraemia was lower in those groups of mice where autointerference was seen however the present study provides no answer as to whether the less pronounced viraemia was a course of reduced mortality

SUMMARY

Autointerference was observed in mice on intraperitoneal inoculation of West Nile virus and was most pronounced in older animals which exhibit a natural resistance against the virus In mice evidencing autointerference the highest level of circulating interferon was found 18 hours after virus inoculation This is in contrast to mice receiving a smaller virus inoculum where the highest level of interferon first appeared 4 days after virus inoculation Treatment with steroids prior to virus inoculation was associated with an inhibition of interferon induction and autointerference was not observed In older mice where autointerference phenomenon was most pronounced viraemia and the amount of circulating interferon were found to be lower than in younger mice

The induction of early interferon in the mice exhibiting auto interference is considered of importance in the reduction of mortality

REFERENCES

1 *Allison A C* Cell mediated immune responses to virus infections and virus induced tumours Brit med Bull 23 60-6 1966

Baron S Mechanism of recovery from viral infection Advances in Virus Res 10 39-64 1963

3 *Baron S Buckler C F Friedman R M & McCloskey R V* Role of interferon during viremia II Protective action of circulating interferon J Immunol 96 17-24 1966

4 *Clarke D H & Casals J* Techniques for haemagglutination and haemagglutination inhibition with arthropodborne viruses Amer J trop Med Hyg 7 561–573 1958

5 *Fantes K H* Purification concentration and physico chemical properties of interferon Interferons Edited by N B Finter North Holland Publishing Company Amsterdam 119–180 1966

6 *Finter N B* Protection of mice by interferon against systemic virus infections. British M J II 981–985 1964

7 *Finter N B* Interferon as an antiviral agent in vivo Quantitative and temporal aspects of the protection of mice against Semliki forest virus Brit J Exper Path 47 361–371 1966

8 *Freundt F.A* Personal communication 1969

9 *Glasgow L A & Habel K* Interferon production by mouse leukocytes in vitro and in vivo J exp Med 117 149–160 1963

10 *Haahr S* The occurrence of virus and interferon in the spleen serum and brain in mice after experimental infection with West Nile virus Acta path microbiol scand 74 445–457 1968

11 *Haahr S* The occurrence of virus and interferon in splean serum and brain in steroid treated mice under experimental infection with West Nile virus. Acta path microbiol scand 75 303–312 1969

12 *Kärber G* Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche Arch f Exper Path u Pharmakol 162 480–490 1931

13 *Maeyer E de & Maeyer Guignard J de* Influence of animal genotype and age on the amount of circulating interferon induced by Newcastle disease virus J gen Virol 2 445–449 1968

14 *Mims C A* Rift Valley fever virus in mice IV Incomplete virus its production and properties Brit J exp Path 37 129–143 1956

15 *Rokutanda H K* Relationship between viremia and interferon production of Japanese encephalitis virus J Immunol 102 662 670 1969

16 *Rytel M W Shope R F & Kilbourne E D* An antiviral substance from Penicillium funiculosum V Induction of interferon by Helenine J exp Med 123 577–584 1966

17 *Sell S & Asofsky R* Lymphocytes and Immunoglobulins Progr Allergy 12 86–160 1968

18 *Schlesinger R W & Frankel J W* Adaption of the New Guinea B" strain of Dengue virus to suckling and to adult Swiss mice Amer J trop Med 1 66–77 1952

19 *Schlesinger R W* Interference between animal viruses The Viruses Edited by F M Burnet and W M Stanley Vol 3 Academic press New York and London p 157–194 1959

20 *Shope R F* An antiviral substance from Penicillium funiculosum IV Inquiry into the mechanism by which Helenine exerts its antiviral effect J exp Med 123 213–227 1966

21 *Smith C F C* Isolation of three strains of type 1 Dengue viru from a local outbreak of the disease in Malaya J Hyg (London) 54 569 580 1956

22 *Smithburn K C* The suspectibility of African wild animals to yellow fever III Pottos and Calagos Amer J trop Med 29 414–423 1949

23 *Theiler M* The virus Yellow Fever Edited by G K Strode McGraw Hill Book Company New York p 81 1951

Acta path microbiol scand 77 413 416 1969

The Institute of Tumour Virus Research the Institute of Medical Microbiology University of Copenhagen Denmark

THE EFFECT OF IMMUNOSUPPRESSIVE THERAPY ON THE MURINE LYMPHOCYTIC CHORIO MENINGITIS VIRUS INFECTION

By

JØRGEN HANNOVER LARSEN

Received 23 iv 69

It has hitherto been demonstrated on a number of occasions that immunosuppressive therapy of adult mice inoculated with lymphocytic choriomeningitis (LCM) virus reduces the lethality rate in the infected mice (16 23) The following immunosuppressive therapy has been used cortisone treatment (17) amethopterine treatment (1 7) and other antimetabolites (7 21) Moreover X ray irradiation (19 23 24) treatment with anti lymphocytic serum (ALS) (6 15 22) or anti mouse IgG (13) have also been used and neonatal thymectomy has also been applied in this infection model (4 18 25)

All these various experiments have given the same clear cut result that the lethality rate of the infection is reduced Following lethal LCM disease large numbers of lymphoid cells are found especially in the meninges and the choroid plexus These infiltrates have been shown to be absent in such treated animals which survive (3 23 24) With regard to the co tolerogenic effect of these procedures the results have been inconclusive Some authors have reported that surviving mice had quite high virus titres for several weeks after the treatment indicating tolerant states but there is no definite information about these conditions except insofar as ALS would seem to be the best immunosuppressive agent in this system also treatment with ALS resulting in tolerance in the treated animals (22)

In order to investigate the effect of cortisone amethopterine (methotrexate) and X irradiation in the LCM/mouse system with respect to the development of tolerance the following experiments were carried out They showed that these treatments only occasionally resulted in a complete and persisting tolerance to the virus (i e no antibody formation and no virus elimination) but often in transient states of incomplete tolerance

---

Requests for reprints should be addressed to J Hannover Larsen Institute of Medical Microbiology 22 Juliane Maries Vej DK 2100 Copenhagen Ø Denmark

The Institute of Tumour Virus Research is supported by grants from the Einar Willumsen's Memorial Fund and the Danish Cancer Society

MATERIALS AND METHODS

LCM virus The LCM virus was the strain which has been used in this laboratory for several years It has been grown by intraperitoneal passages in mice The stock virus consisted of 10 per cent clarified mouse spleen homogenate It was kept at —70 C.

Virus titrations These were performed by intracerebral inoculation into young Swiss mice of 0 03 ml of decimal dilutions of the samples These were either blood *or organ homogenates Four mice per dilution were inoculated* The LD_{50} *titre* was determined after 14 days according to *Kärber*s method (20) and expressed as the reciprocal

The blood samples were taken from the heart in the experiments where the mice were killed Blood samples from surviving animals were taken from the inner canthus of the eye

Complement fixation (CF) This test was performed as described previously (?8) The antigen was made from spleens and livers of tolerant, LCM virus-carrier mice and prepared by an acetone sucrose extraction method (? 5) The control antigen was prepared in the same way from uninfected spleens and livers

Animals Outbred white Swiss mice aged three months weighing about 20 g were used in the experiments Similar mice aged about five to six weeks were used for the virus titrations

Cortisone treatment Cortone® acetate was used and the single dose was 0 8 m It was given subcutaneously every second day for a fortnight, starting on the day of virus inoculation This cortisone dose was chosen after experiments in which groups of adult outbred mice as used in the present experiments were treated with 0 4 mg 0 8 mg and 1 2 mg every second day *A single dose of 0 8 mg* was found to be the maximum tolerated by these animals by this method of administration All the animals were clinically affected by the drug after three to four injections Their pelts were ruffled their skin fragile with occasional ulcerations They were hypokinetic with a congested appearance

Methotrexate treatment Sodium methotrexate® was used in dilution with 2 per cent hydrogen carbonate solution It was administered subcutaneously every second day for a fortnight beginning on the day of the virus inoculation Preliminary experiments using 0 02 mg 0 04 mg and 0 08 mg every second day to adult outbred mice *showed that 0 02 mg repre ented the maximum tolerated dose by this method* of administration These animals were also here clinically affected b the drug after three to four injections

X ray irradiation The mice were irradiated (whole body) by being placed in small holes in a cylinder which rotated under the tube at a distance of 51 cm. The dose rate was 40 r per minute. The physical constants were 175 k 8 mA 0 5 mm Cu The dose was measured by a Victoreen Integron dosimeter After correction for penetration and back scatter the true dose was 430 r All the irradiated animals showed a transient loss of weight during the first week after the treatment. The irradiation was given on the same day as the virus inoculation

RESULTS

The Effect of Cortisone

Two groups each consisting of 30 adult mice were inoculated intracerebrally with 100 and 1 000 LD_{50} LCM virus respectively Both groups were treated with cortisone as described By the end of a fort night 18 and 17 mice from the first and second group respectively had died During the following weeks a number of the remainder died The survivors were followed by individual blood samples during the next six months Then they were killed and their blood spleens and kidneys were titrated for virus contents There were no investigations of antibody production The results of these *observations are listed in* Table 1 This table also contains the results of two groups of simi

TABLE 1

The Lethality and the Course of the Virus Titres in the Blood and Organs of Adult Outbred Swiss Mice Inoculated with LCM Virus and Treated with Cortisone or Methotrexate or Left Untreated

Virus dose	Lethality (14 days)	Treatment	5 weeks after inoc Blood	8 weeks after inoc Blood	11 weeks after inoc Blood	16 weeks after inoc Blood	21 weeks after inoc Blood	6 months after inoc Blood	6 months after inoc Spleen	6 months after inoc Kidney
100 LD_{50}	18/30	Cortisone	4 $>10^{1.0}$§ 7 <10	3 $>10^{0.5}$ 8 <10	–	11 $<10^{0.3}$	11 $<10^{0.3}$	11 <10	11 <10	3 <10 8 $>10^{2.5}$
1000 LD_{50}	17/30	Cortisone	3 $>10^{1.0}$ 9 <10	11 $<10^{0.3}$	–	11 $<10^{0.3}$	11 $<10^{0.3}$	11 <10	11 <10	11 $>10^{\ 3}$
100 LD_{50}	0/10	Methotrexate	10 $>10^{\ 8}$	10 $<10^{\ 5}$	10 $<10^{0.3}$	10 <10	10 $<10^{0.3}$	10 $<10^{0.8}$	10 <10	10 $>10^{\ 3}$
1000 LD_{50}	0/10	Methotrexate	10 $>10^{\ 6}$	10 $<10^{1.0}$	10 $<10^{0.3}$	10 <10	10 <10	10 <10	10 $<10^{0.3}$	1 <10 9 $>10^{2.3}$
100 LD_{50}	8/10	none	1 $10^{0.5}$ 1 <10	2 <10	2 <10	2 <10	2 <10	2 <10	2 <10	2 $>10^{\ 4}$
1000 LD $_{0}$	7/10	none	3 <10	3 <10	3 <10	3 <10	3 <10	3 <10	3 <10	1 <10 2 $>10^{\ 5}$

Number of dead within 14 days/number of inoculated mice
§ Number of mice virus titre

TABLE 2

Virus Titres and CF Titres in the Blood and Organs of Adult Outbred Swiss Mice Inoculated Intraperitoneally with Varying Doses of LCM Virus All the Animals are Treated with Cortisone The Animals were killed at the Time of Investigation

Virus dose	1 month after inoc CF	1 month after inoc Virus titre Blood	2½ months after inoc CF	2½ months after inoc Virus titres Spleen	2½ months after inoc Virus titres Kidney	4 months after inoc CF	4 months after inoc Virus titres Spleen	4 months after inoc Virus titres Kidney	15 months after inoc CF	15 months after inoc Virus titres Blood	15 months after inoc Virus titres Spleen	15 months after inoc Virus titres Kidney
3×10^{8} LD_{50}	64	N T §	64	N T	N T	64	N T	N T	32	N T	N T	Trace
			64	N T	$>10^{5}$	64	N T	$10^{1.8}$	32	N T	Trace	N T
									32	Trace	N T	10^{8}
									16	Trace	N T	$10^{1.5}$
3×10^{4} LD_{50}	64	N T	128	N T	$>10^{5}$	64	N T	Trace	32	N T	N T	$>10^{5}$
			128	Trace	>10	64	N T	$>10^{5}$				
3×10^{2} LD_{50}	64	N T	256	N T	$>10^{5}$	16	N T	N T	32	N T	Trace	$>10^{5}$
			256	N T	N T	32	N T	N T				
3×10^{0} LD_{50}	<4	N T	<4	N T	N T	<4	N T	N T	<4	N T	N T	Trace
			<4	N T	N T	<4	N T	N T	<4	N T	N T	N T
									<4	N T	N T	N T
									<4	N T	N T	N T

Titration on a pool of blood from the whole group

§ N T = no trace

TABLE 3

Virus Titres and CF Titres in the Blood and Organs of Adult Outbred Swiss Mice Inoculated Intraperitoneally with Varying Doses of LCM Virus. The Animals were Killed at the Time of Investigation

Virus dose	1 month after inoc		2½ months after inoc			4 months after inoc			15 months after inoc			
		Virus titre		Virus titres			Virus titres			Virus titres		
	CF	Blood	CF	Spleen	Kidney	CF	Spleen	Kidney	CF	Blood	Spleen	Kidney
3×10^{5} LD$_{50}$	64	N T§	64	N T	N T	64	$10^{1.3}$	>10 5	16	N T	Trace	N T
			64	N T	N T	32	Trace	N T	32	N T	N T	N T
			64	N T	N T	64	N T	N T	16	N T	Trace	N T
3×10^{4} LD$_{50}$	32	N T	64	N T	N T	64	N T	N T	16	N T	N T	N T
			64	N T	N T	32	N T	N T	16	N T	N T	N T
			128	Trace	>10 5	64	N T	N T	32	N T	N T	N T
3×10^{3} LD$_{50}$	64	N T	8	Trace	N T	64	N T	N T	32	N T	N T	N T
			64	Trace	N T	64	N T	N T	32	N T	N T	>10
			32	N T	N T	64	N T	N T	64	N T	N T	N T
3×10^{2} LD$_{50}$	8	N T	128	N T	N T	<4	N T	N T	<4	N T	N T	N T
			<4	N T	N T	64	$10^{1.3}$	N T	<4	N T	N T	N T
			<4	N T	N T	<4	N T	N T	<4	N T	N T	N T

Titration on a pool of blood from the whole group

§ N T = no trace

larly inoculated mice which were left untreated Only two and three mice respectively survived (out of ten) and were followed

The results show a moderate decrease in the lethality rate in the cortisone-treated groups The viraemia was eliminated after five to eight weeks in most of the mice After six months the virus had been eliminated from the blood and spleens but in most of the animals—both those treated with cortisone and the controls—a high virus content still persisted in the kidneys None of these animals developed tolerance

In order to see whether tolerance could be induced in the adult cortisone treated mouse by means of large amounts of virus the following experiments were performed Groups of adult mice (10 mice per group) were inoculated intraperitoneally with 3 LD_0 3 × 10 LD_{50} 3 × 10^4 LD_0 and 3 × 10^6 LD_5 All the animals were treated with cortisone At intervals hereafter one to four animals per group were killed Their blood was titrated for CF antibodies and their organs for virus content The results of these observations are recorded in Table 2 A total of 26 mice were tested 14 died during the long observation period

For comparison the results of similarly inoculated groups of mice which were left untreated are recorded in Table 3

No distinct differences were found between the treated and the untreated groups as regards the virus elimination and the antibo formation No states of tolerance developed in the cortisone treat groups not even after the large virus dose In the treated groups fo mice still had high virus content in the kidneys after 15 months The were similar findings in only one mouse in the untreated group

The Effect of Methotrexate

A series of experiments on similar lines to the cortisone experimen were performed using methotrexate

After intracerebral inoculation of 100 and 1 000 LD LCM vir into two groups of ten mice each the lethality in the animals treate with methotrexate was nil after a fortnight The results from thes groups are also listed in Table 1 High viraemia was still found fou weeks after the virus inoculation but it disappeared during the follow ing weeks All mice investigated after six months had high viru content in the kidneys

After intraperitoneal inoculation of 3 LD_{50} 3 × 10 LD_0 3 × 10 LD_0 and 3 × 10^6 LD_{50} (see Table 4) the CF titres were lower afte one month than in the corresponding cortisone treated and untreate groups (Tables 2 and 3) The virus was eliminated at a slower rat from the spleens and the high renal virus content was found muc more frequent in this experiment It was found in all investigated ani mals except in the group inoculated with the low virus dosage Only

TABLE 4

Virus Titres and CF Titres in the Blood and Organs of Adult Outbred Swiss Mice Inoculated Intraperitoneally with Varying Doses of LCM Virus. All the Animals are Treated with Methotrexate. The Animals were Killed at the Time of Investigation

Virus dose	1 month after inoc		2½ months after inoc			4 months after ino			15 months after inoc			
		Virus titre		Virus titres			Virus titres			Virus titres		
	CF	Blood	CF	Spleen	Kidney	CF	Spleen	Kidney	CF	Blood	Spleen	Kidney
3×10⁶ LD	9	>10	<4 <4	>10 5 N T	>$10^{2.5}$ >10	32 <4	N T § N T	>10 5 >10 5	<4 <4	Trace N T	$10^{1.5}$ $10^{1.8}$	>10 >10 5
3×10⁴ LD	<4	>10 5	32 8	10 N T	>$10^{3.5}$ >10 5	<4 <4	N T N T	>$10^{2.6}$ >$10^{2.5}$	16 8 16 <4	N T N T N T >10 5	N T N T $10^{1.0}$ >$10^{2.5}$	>10 5 $10^{1.5}$ >10 5 >10^{2}
3×10² LD₅₀	<4	>10 5	16 <4	$10^{1.8}$ $10^{1.0}$	>10 5 >10 5	16 <4	10 3 N T	>$10^{2.5}$ >$10^{2.5}$	16 32 8	10 10 N T	Trace N T N T	>10 5 >10 5 $10^{1.3}$
3×10⁰ LD₅₀	32	>10 5	<4 128	N T N T	N T N T	<4 <4	N T N T	N T N T	<4 <4 16	N T N T N T	N T $10^{1.0}$ Trace	N T N T N T

Titration on a pool of blood from the whole group
§ N T = no trace

(nos 1 and 8 in the 100 LD_{50} group) no antibodies were found at any time despite high viraemia at the beginning of the observation period

DISCUSSION

In the present LCM virus/mouse system the intracerebral inoculation is the most sensitive means of estimating the lethal effect of the virus (cf LD_{50} determinations) In the present experiments in which the intracerebral route has been used a clear cut effect of the three immunosuppressive procedures on the lethality rate has been found (Tables 1 and 5) This effect is most pronounced in the methotrexate treatment where the lethality rate is nil Similarly low lethality rate has also been found in therapy with ALS or rabbit anti mouse IgG (6 13) In the present experiments no histopathological examinations were made It might be assumed that the lymphoid infiltrates in the meninges and choroid plexus—possibly provoked by the virus as localized immunological reactions of the host to the virus and considered to be the cause of death of the animals (3 23 24)—were also abolished to some extent by the therapy resulting in survival of the animals

Before the discussion on the development of tolerance the author s criteria for complete and incomplete tolerance will be stated These criteria are based on observations of thousands of tolerant animals The criteria for a complete tolerance in the present mouse/virus system are 1) The presence of a long lived content of virus in the animal as expressed by virus titres of $\leq 10^{-3}$ in the blood 2) The absence of measurable amounts of CF antibodies titres <4 At no time has any immunological disease or wasting been found among mice fulfilling these criteria provided that the tolerance was induced by *inoculation of a sufficient virus dose within the first 18 hours of life* (11 27) The incompletely tolerant states in this system may take various forms 1 The virus titres in the blood are at virus carrier level (*i e* $\leq 10^{-3}$) together with CF antibody formation 2 The virus titres are below virus carrier level without CF antibody formation 3 A combination of both 1 and 2 These incompletely tolerant states are usually transient and often result in a weakening of the animal (10 14)

In the present experiments no development of tolerance was found in the intracerebrally inoculated mice treated with cortisone or methotrexate The virus elimination was somewhat more delayed in the *methotrexate treated groups indicating a suppression of this* mechanism The phenomenon of maintained high virus titres in the kidneys was very pronounced in these groups even after six months This indicates that the virus eliminating mechanism did not function in the kidneys As this virus elimination is most probably mainly dependent on a cell mediated immune response of the host to LCM

virus (8 10 11 12) this would suggest the existence in the kidneys of an immune barrier to this immune response The same phenomenon has previously been found in the same virus/mouse system where the tolerant state has been abolished by adoptive immunization (8 26 27)

In the irradiated mice (Table 5) the virus elimination was also suppressed in most of the animals but antibodies were formed in quite high titres However in only two of the mice had the immune response been suppressed completely with the resultant development of a complete tolerance This occurred in mice nos 1 and 5 in the 100 LD_{50} group The latter had in addition a transient phase of antibody formation In mouse no 8 in the same group there was virus elimination without any humoral immune response This part of the immune response must have been suppressed while the other part—the cell mediated—was not i e an incompletely tolerant state presumably indicating a state of split tolerance This phenomenon has also been found in some mice inoculated at an age of 2 to 9 days with LCM virus (10) In several of the mice in these irradiated groups it was found that high virus titres and CF titres were present at the same time This presumably also indicated states of split tolerance

The experiments with intraperitoneal inoculation of varying virus doses (Tables 2 3 and 4) illustrate that large amounts of this virus strain did not harm the mice As can be seen in the untreated groups (Table 3) the virus was eliminated from the blood after four weeks and antibodies were formed at this time Only few mice continued to have high renal virus titres No states of tolerance were found in any of these untreated mice In the cortisone treated groups the virus was also eliminated from the blood after four weeks but the high renal (and to a lesser degree—splenic) virus titres were more pronounced The antibody titres were by and large at a higher level than in the untreated groups No states of tolerance were found in any of the mice in these groups

In the methotrexate treated groups high viraemia and low or no antibody titres were found after four weeks After 2½ months virus elimination was functioning in most of the mice This means that the virus eliminating process (i e the cell mediated immune response) had been suppressed up to this time The antibody titres were at a very low level indicating a simultaneous suppression of the humoral response The suppression persisted in only two mice resulting in a completely tolerant state after 15 months The phenomenon of the persisting high renal virus titres is found in almost all mice in these groups except for the low virus inoculated groups

The low virus dose of 3 LD_{50} had only a slight or no immunogenic effect in all experiments this may presumably be explained by little or no multiplication of the virus after this dose There was no great difference between the results in mice inoculated with the very varied

(nos 1 and 8 in the 100 LD_{50} group) no antibodies were found at any time despite high viraemia at the beginning of the observation period

DISCUSSION

In the present LCM virus/mouse system the intracerebral inoculation is the most sensitive means of estimating the lethal effect of the virus (cf LD_{50} determinations) In the present experiments in which the intracerebral route has been used a clear cut effect of the three *immunosuppressive procedures on the lethality rate has been found* (Tables 1 and 5) This effect is most pronounced in the methotrexate treatment where the lethality rate is nil Similarly low lethality rate has also been found in therapy with ALS or rabbit anti mouse IgG (6 13) *In the present experiments no histopathological examinations* were made It might be assumed that the lymphoid infiltrates in the meninges and choroid plexus—possibly provoked by the virus as localized immunological reactions of the host to the virus and considered to be the cause of death of the animals (3 23 24)—were also abolished to some extent by the therapy resulting in survival of the animals

Before the discussion on the development of tolerance the author s criteria for complete and incomplete tolerance will be stated These criteria are based on observations of thousands of tolerant animals The criteria for a complete tolerance in the present mouse/virus system are 1) The presence of a long lived content of virus in the animal as expressed by virus titres of ≦10 ³ in the blood 2) The absence of measurable amounts of CF antibodies titres <4 At no time has any immunological disease or wasting been found among mice fulfilling these criteria provided that the tolerance was induced by inoculation of a sufficient virus dose within the first 18 hours of life (11 27) The incompletely tolerant states in this system may take various forms 1 The virus titres in the blood are at virus carrier level (*i e* ≦10 ³) together with CF antibody formation 2 The virus titres are below virus carrier level without CF antibody formation 3 A combination of both 1 and 2 These incompletely tolerant states are usually transient and often result in a weakening of the *animal* (10 14)

In the present experiments no development of tolerance was found in the intracerebrally inoculated mice treated with cortisone or methotrexate The virus elimination was somewhat more delayed in the methotrexate treated groups indicating a suppression of this mechanism The phenomenon of maintained high virus titres in the kidneys was very pronounced in these groups even after six months This indicates that the virus eliminating mechanism did not function in the kidneys As this virus elimination is most probably mainly dependent on a cell mediated immune response of the host to LCM

REFERENCES

Barlow J I & Hotchin J Induction of persistent tolerant infection with lymphocytic choriomeningitis virus in adult mice by amethopterin treatment A R N Y St D p Hlth 39 19 2

Clarke D H & Casals J Techniques for hemagglutination and hemagglutination inhibition with arthropod borne viruses Amer J trop Med Hyg 7 561-573 1958

Collins D N Weigand H & Hotchin J The effect of pretreatment with x rays on the pathogenesis of lymphocytic choriomeningitis in mice II The pathological histology J Immunol 87 682-687 1961

East J Parrott D M V & Seamer J The ability of mice thymectomized at birth to survive infection with lymphocytic choriomeningitis virus Virology 22 160-162 1964

Gresikova M & Casals J A simple method of preparing a complement fixing antigen for lymphocytic choriomeningitis virus Acta virol 7 380 1963

Gledhill A W Protective effect of anti lymphocytic serum on murine lymphocytic choriomeningitis Nature (Lond) 214 178-179 1967

Haas V H & Stewart S E Sparing effect of amethopterin and guanazolo in mice infected with the virus of lymphocytic choriomeningitis Virology 2 511 516 1956

8 Hannover Larsen J Den lymphocytære choriomeningitis virusinfektion hos musen Thesis University of Copenhagen 1968

9 Hannover Larsen J Studies on immunological tolerance to LCM virus 9 Induction of immunological tolerance to the virus in the adult mouse Acta path microbiol s and 73 105 114 1969

10 Hannover Larsen J On the induction of immunological tolerance to a self r producing antigen Immunology 16 15-23 1969

11 Hannover Larsen J Immunological tolerance and the significance of cell mediated immunity in virus infections Dan med Bull 16 145-154 1969

12 Hannover Larsen J Development of humoral and cell mediated immunity to lymphocytic choriomeningitis in the mouse J Immunol 102 941 946

13 Hannover Larsen J Unpublished work 1969

14 Hirsch M S Murphy F A & Hicklin M D Immunopathology of lymphocytic choriomeningitis virus infection of newborn mice Antithymocyte serum effects on glomerulonephritis and wasting disease J exp Med 127 757-763 196

15 Hirsch M S Murphy F A Russe H P & Hicklin M D Effects of anti thymocyte serum on lymphocytic choriomeningitis (LCM) virus infection in mice Proc So exp Biol (N Y) 125 980-983 1967

16 Hotchin J The biology of lymphocytic choriomeningitis infection Virus induced immune disease Cold Spr Harb Symp quant Biol 27 479-499 1962

17 Hotchin J & Cinits M Lymphocytic choriomeningitis infection of mice as a model for the study of latent virus infection Canad J Microbiol 4 149-163 1958

18 Hotchin J & Sikora F Protection against the lethal effect of lymphocytic choriomeningitis virus in mice by neonatal thymectomy Nature (Lond) 202 14-215 1964

19 Hotchin J & Weigand H The effect of pretreatment with x rays on the pathogenesis of lymphocytic choriomeningitis in mice I Host survival virus multiplication and leukocytosis J Immunol 87 675-681 1961

20 Kärber G Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche Naunyn Schmiedeberg s Arch exp Path Pharmak 162 480-483 1931

21 Levy H B & Haas V H Alteration of the course of lymphocytic choriomeningitis in mice by certain antimetabolites Virology 5 401-407 1958

22 Lundstedt C & Volkert M Studies on immunological tolerance to LCM virus 8 Induction of tolerance to the virus in adult mice treated with anti lymphocytic serum Acta path microbiol s and 71 471-480 1967

23 Rowe W P Studies on pathogenesis and immunity in lymphocytic choriomeningitis infection of the mouse Nav med. Res Inst. Rep 12 167-220 1954

24 *Rowe W P* Protective effect of pre irradiation on lymphocytic choriomeningitis infection in mice Proc Soc exp Biol (NY) *92* 194–198 1956
25 *Rowe W P Bloch P H & Levey R H* Protective effect of neonatal thymectomy on mouse LCM infection Proc Soc exp Biol (NY) *114* 248–251 1963
26 *Volkert M & Hannover Larsen J* Studies on immunological tolerance to LCM virus 3 Duration and maximal effect of adoptive immunization of virus carriers Acta path microbiol scand *60* 577–587 1964
27 *Volkert M & Hannover Larsen J* Immunological tolerance to viruses Progr med Virol 7 160–207 1965
28 *Volkert, M Hannover Larsen J & Pfau C J* Studies on immunological tolerance to LCM virus 4 The question of immunity in adoptively immunized virus carriers Acta path microbiol scand *61* 268–282 1964

cta path microbiol s and 77 447–45(1969

aboratory of Oral Microbiology Department of Microbiology The Gade Institute
nd Institute of Periodontology Schools of Medicine and Dentistry University of
Bergen Norway

IMMUNOCHEMICAL STUDIES OF ORAL FUSOBACTERIA

3 Purification of a Group Reactive Precipitinogen

By

TORE KRISTOFFERSEN

Received 23 iv 69

The major precipitinogens found in the *Fusobacterium* strain F1 isolated in our laboratory have been reported earlier (3) Some of the characteristics of the precipitinogens were also described (3) One of the precipitating antigens provisionally termed Precipitinogen 2 was found to be present in all of 20 strains of oral fusobacteria studied This precipitinogen was heat labile and was destroyed by 45 per cent phenol by digestion with pepsin and pronase and by oxidation with periodate Apparently Precipitinogen 2 is a group specific antigen containing protein and possibly a carbohydrate component (3)

Attempts have been made to purify this substance Some of our experiments along with a purification procedure for Precipitinogen 2 will be reported in the present paper

MATERIALS AND METHODS

Strains The methods for *isolation and characterization* of fusobacteria have been described (3) Strain F1 was selected to serve as source of Precipitinogen 2 in the present study Extracts from this microorganism showed high titres of Precipitinogen 2

For mass cultivation the bacteria were grown in fluid Brain Heart Infusion broth (Difco) usually for 4 days and harvested by centrifugation *After harvesting* the organisms were washed twice with sterile distilled water and stored as a paste at —25 C until use.

Disruption of bacteria was achieved by 5 passages of washed bacteria through the X Press (1)

Rabbit immune sera were prepared as described in (3) Antisera against the type strain ATCC 10 953 or against the strain F 30 a isolated in our laboratory precipitated only line 2 in agar with most extracts from strain F 1 These antisera were used to follow the titres of Precipitinogen 2 during extraction and fractionation procedures Antiserum against strain F 1 and antisera against 8 additional strains of *Fusobacteria* were used to check for the presence of precipitating antigens other than Precipitinogen 2 in the fractions

Agar double diffusion tests were performed as previously described (3) Diffusion

These investigations have been supported by grants from *L Meltzers Høgskolefond* and from the *Norwegian Research Council for Science and the Humanities*

and precipitation took place at room temperature Whenever the objective of the test was to follow the agar precipitation titre during fractionation and purification processes two fold serial dilutions of the antigenic fraction were put into the peripheral wells and undiluted antiserum in the central well In these instances readings were made after 24 hours Otherwise readings were made after 1 2 and 3 days

The methods used for *ring test precipitation immunoelectrophoresis* and *paper electrophoresis* were those described in (3) Electrophoresis strips were stained with amido black only

For *gel filtration* with Sephadex G 25 G 50 G 100 and G 200 (AB Pharmacia Uppsala Sweden) columns were packed largely as described by *Flodin* (2) The ratio diameter height of the columns varied from 1 10 to 1 15 Columns of Sephadex G 25 Coarse used for desalting were calculated (5) to give complete separation of the active material and salts in the sample volume to be desalted Experiments to test the efficiency of the columns for desalting were also run

Columns for *ion exchange chromatography* were prepared from DEAE cellulose (DEAE SS Serva Heidelberg) The ratio diameter height of the columns varied from 1 5 to 1 10

Preparative ultra centrifugation was performed in a Beckman Spinco Model L ultracentrifuge in 10 ml tubes

The method employed for *ultrafiltration* was similar to that described in (6) except that a glass cylinder 2 5 × 120 cm equipped with a vacuum outlet was used instead of a desiccator The dialysis bag (Arthur Thomas Philadelphia) had an extended diameter of 1 inch and was connected to a sample reservoir The volume of a solution could be reduced by more than 25 ml per hour at 4 C in this way

Concentration with Sephadex (2) was achieved by adding amounts of dry Sephadex G 25 Coarse calculated to give a two fold concentration After 15 minutes the active material was removed from the swollen gel by pressure filtration

Proteins were determined by the Folin Ciocalteau phenol method according to *Lowry et al* (4) with bovine serum albumin as standard

Neutral sugars were estimated by the sulphuric acid orcinol method (9) Since preliminary chromatographic examinations of acid hydrolysates of our crude extracts and fractions had indicated that glucose was the dominant neutral sugar present glucose was used as standard

Total free lipids were estimated largely as described by *Sperry* (8) *Fatty acid esters* were determined by the method described by *Snyder & Stephens* (7) Tripalmitin was used as standard

Total activity is defined as the precipitation titre in agar multiplied by the volume in ml

Specific activity is defined as the precipitation titre in agar divided by the Folin protein value expressed in mg/ml

Unless otherwise stated all fractionation procedures were carried out at room temperature

EXPERIMENTS AND RESULTS

A Preliminary Experiments

Some results from preliminary experiments which provided a foundation for a purification procedure will be reported in some detail

Extraction A number of experiments were carried out to determine optimal conditions for extraction of Precipitinogen 2 from microorganisms Disintegration of the bacteria prior to extraction invariably gave superior results The yield was increased as much as four fold by crushing the organisms prior to extraction The specific activity was the same with crushed and whole bacteria

Crushed microorganisms were extracted with different buffers at various temperatures Extraction was continued for 24 hours and the supernatant after centrifugation at 20 000 x *g* for 30 minutes was col

lected Extraction with 0 05 M phosphate buffer pH 7 4 at 4 C was found to give good results

Stability of extracts Crude buffer extracts could be stored for several days at 4 C without apparent loss of Precipitinogen 2 After a certain degree of purification had been achieved the material became very unstable however Clear solutions containing Precipitinogen 2 became opaque flocculent precipitates appeared and the precipitating ability disappeared or was greatly reduced These alterations were observed particularly when solutions of partially purified material were subjected to prolonged dialysis against tap or distilled water Concentration of purified or partially purified solutions under reduced pressure at temperatures well below 40 C resulted in a rapid and eventually complete loss of activity of Precipitinogen 2 Freezing and thawing had a similar effect Lyophilization of dialyzed solutions rendered the material nearly insoluble in aqueous media even at strongly alkaline pH The denaturation and inactivation appeared to be irreversible

Precipitinogen 2 could be at least partially protected from denaturation during purification procedures by including 0 001 M ethylene diaminetetraacetate (EDTA) and 0 005 M 2 mercaptoethanol (ME) in the buffer solutions used Partially purified solutions could be stored for several days at 4 C in this way without serious loss of serological activity

Fractional precipitation Precipitation with acid precipitation with varying concentrations of Rivanol (Hoechst, Frankfurt a M) and fractional precipitation with organic solvents at room temperature and at 4 C gave poor yields and little or no purification Precipitinogen 2 could however be precipitated in good yields from crude extracts or partially purified solutions with 3 volumes of acetone at −24 C Acetone precipitates were collected by rapid centrifugation in a refrigerated centrifuge dried *in vacuo* and ground to a fine powder Such powders could be washed with ethanol ether (2 1) at −24 C without apparent damage to the active material

Dialyzed and lyophilized crude buffer extracts from strain F1 contained 10–15 per cent of free lipids and approximately 10 per cent of fatty acid esters Some of the lipid material was not readily disposed of during later stages of the purification procedure Treatment with acetone and ethanol ether at −24 C not only served to remove free lipid but also removed a considerable amount of contaminating proteins The purification in this respect was almost three fold Equally good results were obtained when a suspension of crushed microorganisms in distilled water was subjected to the acetone and ethanol ether treatments at −24 C prior to extraction for 24 hours with 0 05 M phosphate buffer pH 7 4 The latter procedure had the additional advantage that it involved the handling of smaller volumes Five grams of bacteria treated in this manner did not contain measureable amounts of free lipid

Almost 80 per cent of Precipitinogen 2 was precipitated from crude extracts between 40 and 70 per cent saturation with ammonium sulphate and a three fold purification could be achieved Most of the Freeman type hapten (3) and the endotoxic lipopolysaccharide (3) were also found in the 40–70 per cent ammonium sulphate fraction The 40–50 50–60 and 60–70 per cent fractions were found to be similar with regard to both total activity and specific activity

Column chromatography Precipitinogen 2 could be adsorbed to columns of DEAE cellulose equilibrated with suitable buffers when partially purified extracts were applied to the colums The material was eluted in good yields by gradient or stepwise elution with increasing salt concentrations A column of DEAE cellulose equilibrated with 0 02 M phosphate buffer pH 6 3 containing 0 001 M EDTA and 0 005 M ME was found to give good results Unless the material applied to the columns had been sufficiently purified certain contaminants notably polysaccharide materials identifiable as Freeman type hapten and endotoxic lipopolysaccharide were eluted from the columns together with Precipitinogen 2

Ultracentrifugation and gel filtration Most of the endotoxic lipopolysaccharide could be removed prior to ion exchange chromatography by ultracentrifugation at 100 000 x *g* for 1 hour By this procedure the lipopolysaccharide was precipitated as a gelatinous material Gel filtration of the supernatant through columns of Sephadex G 200 separated Precipitinogen 2 from the Freeman type hapten and residual lipopolysaccharide fairly well Fractions from columns of Sephadex G 200 which gave only the line corresponding to Precipitinogen 2 in agar contained about 60 per cent of the activity of the sample applied to the column and the specific activity was about 5 times higher

Concentration and desalting Attempts to concentrate and desalt solutions of purified Precipitinogen 2 prior to lyophilization generally resulted in severe denaturation and loss of serological activity Even with ultrafiltration for 6 hours at 4 C considerable precipitation with concomitant loss of activity occurred Concentration with *Sephadex G 25 Coarse gave the best results A four fold concentration* could be obtained in two steps with 60–80 per cent yield of active soluble material The specific activity was unchanged

The time needed for desalting by dialysis could be shortened considerably by increasing the surface area per unit volume of solution and by providing for stirring inside and outside the dialysis bag However some precipitation and loss of activity still occurred *On* desalting by gel filtration on columns of Sephadex G 25 Coarse some of the active material tended to precipitate in the gel bed The recovery of active soluble material by this procedure was usually about *70 per* cent The product obtained after freeze drying of material *desalted*

in this manner was white and fluffy soluble in aqueous media at neutral pH and had the same specific activity as before desalting

B Purification Procedure

On the basis of the preliminary experiments a procedure for the purification of *Precipitinogen 2 could be adopted* It comprised the following steps

1) Preparation of an acetone powder from crushed microorganisms Further removal of lipids with ethanol ether (2:1)
2) Extraction of dried defatted microorganisms *with 0.05 M phos*phate buffer pH 7.4 at 4 °C
3) *Fractional precipitation* with ammonium sulphate All buffers used after this step contained 0.005 M MF and 0.001 M EDTA
4) Ultracentrifugation at 100 000 x *g*
5) Gel filtration on Sephadex G 200
6) Ion exchange chromatography on DEAE cellulose at pH 6.3

A detailed account of the purification procedure will be presented by *reporting the purification of one batch of* Precipitinogen 2 Some details of the purification procedure are shown in Table 1

TABLE 1

Purification Steps for Precipitinogen 2

Purification steps	Volume ml	Agar precipitation titre serum *F30a* (reciprocal)	Recovery per cent	Protein mg/ml	Specific activity
Extract from crushed "defatted dried bacteria	210	8	100	4.96	1.6
Ammonium sulphate precipitate	40	32	76	12.50	2.7
Ultracentrifugation and gel filtration on G 200	100	8	48	0.57	14.0
Ion exchange chromatography	190	4	45	0.16	25.0

In this experiment 40 g (wet weight) *of crushed microorganisms* strain F1 were suspended in 50 ml of distilled water at 4 °C This slurry was poured slowly into 3 volumes of acetone prechilled to -24 °C and extracted with gentle stirring at this temperature for about 5 minutes The microorganisms were collected by rapid centrifugation in a re*frigerated centrifuge* at 4 °C and dried *in vacuo* overnight The dried

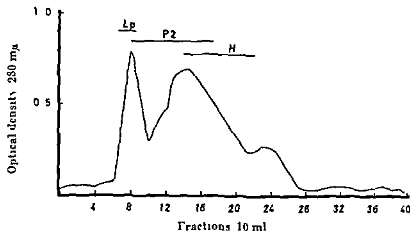


Fig 1

Purification of Precipitinogen 2 by gel filtration on Sephadex G 200 at pH 6 3
Lp = Endotoxic lipopolysaccharide
P2 = Precipitinogen 2
H = Freeman type hapten

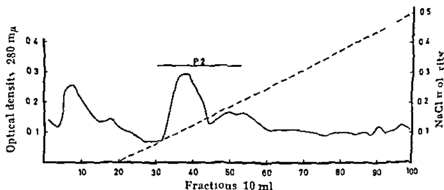


Fig 2

Purification of Precipitinogen 2 by column chromatography on DEAE cellulose at pH [illegible] 3
P2 = Precipitinogen 2

material was ground to a fine powder and extracted for 10 minutes with 100 ml ethanol ether (2 1) at —24 C The defatted microorganisms were again dried *in vacuo* after rapid centrifugation The weight of the dried defatted microorganisms was approximately 6 g This powder was suspended in 200 ml of 0 05 M phosphate buffer pH 7 4 by the use of a Servall Omni Mixer at low speed for 1 hour Extraction was then allowed to proceed overnight at 4 C The mixture was then centrifuged at 25 000 x *g* for 30 minutes the bacterial residue was washed with 30 ml of the same buffer and the supernatants were combined The total volume of the extract was 210 ml It contained 0 7 *mg of neutral sugars per ml The agar precipitation titre* against an anti serum to strain F 30a was 8 and the specific activity was 1 6 To this

solution was added 50 g ammonium sulphate at room temperature When the salt had dissolved the solution was left at 4 C for 1 hour The precipitate after centrifugation at 12 000 x g for 30 minutes was discarded and another 67 g of ammonium sulphate was added to the supernatant with stirring The mixture was left at 4 C for 1 hour after the salt was dissolved and centrifuged as before The precipitate was dissolved in 0 02 M phosphate buffer pH 6 3 containing 0 001 M EDTA and 0 005 M ME and made up to 40 ml in the same buffer Approximately 75 per cent of the precipitating activity was recovered in this fraction and the specific activity was twice as high as in the original extract

This material was subjected to ultracentrifugation at 100 000 x g for 1 hour The supernatant was drawn off and the gelatinous deposits were discarded

Twenty ml of the supernatant fluid was applied to each of two columns 3 × 45 cm packed with Sephadex G 200 The columns had been equilibrated with 0 02 M phosphate buffer pH 6 3 containing 0 005 M of ME and 0 001 M of EDTA Elution was carried out with the same buffer The flow rate was approximately 20 ml per hour and 10 ml fractions were collected Some data from one of the Sephadex fractions are shown in Fig 1 A small amount of lipopolysaccharide material appeared in the eluate after 60 ml and could be found in the next two 10 ml fractions Precipitinogen 2 appeared in fractions 8 through 16 with a peak activity (ring test titre) in fraction number 10 The maximal activity of Precipitinogen 2 did not correspond to any of the three peaks of U V absorbing material observed Material precipitating a line in agar corresponding to the Freeman type hapten appeared from fraction number 14 Fractions number 9 through 12 from both columns precipitated only one line in agar against homologous and heterologous antisera These fractions were pooled They contained more than 40 per cent of the original activity of Precipitinogen 2 and the purification obtained by gel filtration was about five fold The ultraviolet absorption spectrum however showed a peak of absorbancy at 260 mμ indicating the presence of relatively large amounts of nucleoproteins in the fractions

After purification by gel filtration the material was applied directly to a column of DEAE cellulose 2 8 × 25 cm (Fig 2) The column had been equilibrated with the same buffer that was used for gel filtration The eluate was collected in 10 ml fractions After the material had entered the column 200 ml of the same buffer was passed through Some U V absorbing material without precipitating activity passed through the column in these fractions A linear gradient to 0 5 M NaCl in the starting buffer was subsequently used for elution of the column The active material was eluted almost quantitatively between 0 1 and 0 2 M NaCl No other precipitinogens could be detected in these fractions The maximal activity of Precipitinogen 2 corresponded to a

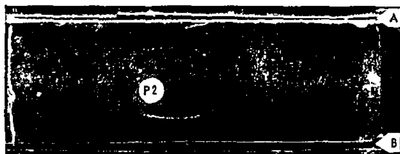


Fig 3

Immunoelectrophoresis of the purified Precipitinogen 2

P2 = Purified Precipitinogen 2 1 mg/ml in phosphate buffered saline
A = Antiserum to *Fusobacterium polymorphum* strain Fev1
B = Antiserum to strain Fl

single and fairly symmetrical peak When the NaCl molarity was raised above 0 6 materials with high optical density particulary at 260mμ appeared in the eluates but no active material was detected

The specific activity was increased about twofold by ion exchange chromatography The increase during the entire purification procedure not including the initial treatment of the bacteria with acetone and ethanol ether was about fifteen fold The content of neutral sugars in the unconcentrated material obtained from the DEAE cellulose column was too low to be measured accurately

The pooled active fractions were concentrated to approximately 40 ml with Sephadex G 25 Coarse desalted by passage on a column of the same material 5 × 45 cm and lyophilized The yield was 21 mg of a white fluffy material which gave clear solutions in water at neutral and alkaline pH in concentrations up to a least 2 mg/ml

The ultraviolet absorption spectrum of a 0 2 per cent solution of the material in buffered saline showed no peak or shoulder at 260mμ

Only one line was formed in agar double diffusion tests and immunoelectrophoresis experiments when a solution containing 1 mg/ml was used as antigen against homologous and heterologous antisera (Fig 3) When samples containing approximately 1 0 mg of protein (Folin value) were passed through columns 1 × 25 cm of Sephadex G 25 G 100 and G 200 equilibrated with the buffer used previously for gel filtration the material was eluted as a single symmetrical peak

On paper electrophoresis in buffers with pH 6 6 (phosphate buffer I = 0 1) pH 8 6 (veronal buffer I = 0 1) and pH 8 9 (High Resolution Buffer LKB Stockholm) the material moved slowly and as a single band towards the anode

DISCUSSION

Although the agar precipitation titre utilizing two fold serial dilutions gave reproducible results admittedly this method can only give rough

estimates of the total activity and specific activity of an extract and more subtle differences between two fractions can not be detected The estimates of recovery and specific activity presented in Table 1 therefore can only be regarded as approximates Ring test titres of Precipitinogen 2 were usually 4 times higher than agar precipitation titres and more exact estimates of the serological activity could be obtained However since non specific precipitation was produced with several of our antisera at pH 6 3 dialysis of such fractions would be necessary The ring test with appropriate controls therefore was used only for some of the introductory experiments

The instability in solution of Precipitinogen 2 has presented considerable difficulties during purification experiments It appeared from introductory experiments that several mechanisms might be responsible for the denaturation and inactivation of Precipitinogen 2 No attempt has been made to study this problem further nor has the possible stabilizing effect of EDTA and ME when used separately been studied Examination of residual activity of Precipitinogen 2 in the fractions which have been discarded during the purification process suggests that very little of the activity is lost through denaturation by the methods employed

The extraction of the microorganisms first with acetone and then with ethanol ether (2 1) appeared to be very effective in removing free lipids The extract obtained from defatted microorganisms also contained less contaminating protein than extracts obtained by direct extraction of bacteria If this initial step is taken into account the purification of Precipitinogen 2 achieved through the entire process amounts to about 75 fold

Precipitation with ammonium sulphate was studied in some detail but no further purification than that reported could be achieved without considerable loss of active material

Ultracentrifugation at 100 000 × g for 1 hour was a simple and convenient method for removing high molecular contaminants such as lipopolysaccharide complexes The loss of Precipitinogen 2 by this procedure was negligible

Gel filtration on Sephadex G 200 gave somewhat better results when smaller samples were applied to the columns Increasing the column volume had the same effect The procedure described has proved to be convenient for a standard preparative technique A considerable purification was obtained Furthermore the salt concentration in the fractions containing Precipitinogen 2 was adjusted to a level which made it possible to adsorb the material to colums of DEAE cellulose directly without including a dialysis step

The recovery from the DEAE cellulose columns was over 90 per cent and the specific activity was increased about two fold There appeared to be no indication that contaminating substances were eluted from the ion exchange columns along with Precipitinogen 2

The losses of active material during the final concentration and desalting procedures were considerable However these procedures with subsequent lyophilization resulted in a purified serologically active product which could be stored for months for the purpose of serological investigations

The techniques employed resulted in a product which was free from contaminating precipitinogens It also was free from other contaminants detectable by the gel filtration and electrophoresis experiments performed Additional tests for the homogeneity and purity of the product have been difficult to perform particularly because of the instability of Precipitinogen 2 in solution

Investigations on some chemical and serological properties of Precipitinogen 2 are in progress

SUMMARY

A method for the purification of a group specific precipitinogen from oral fusobacteria has been described The purification procedure involves the following steps

1 Extraction from crushed defatted and dried microorganisms
2 Fractional precipitation with ammonium sulphate
3 Ultracentrifugation and gel filtration
4 Ion exchange chromatography

The product obtained was homogeneous in agar precipitation immunoelectrophoresis gel filtration and paper electrophoresis experiments

REFERENCES

1 *Edebo L* A new press for the disruption of microorganisms and other cells J biochem microbiol Technol Engng 2 453–479 1960

2 *Flodin P* Dextran gels and their application in gel filtration Pharmacia Uppsala 1962

3 *Kristoffersen T* Immunochemical studies of oral fusobacteria I Major precipitinogens Acta path microbiol scand 77 235–246 1969

4 *Lowry O H Rosebrough N J Farr A L & Randall R J* Protein measurement with the Folin phenol reagent J biol Chem *193* 265–275 1951

5 *Morris C J O R & Morris P* Separation methods in biochemistry Pitman & Sons London 1964 p 385–394

6 *Peterson E A & Sober H A* Column chromatography of proteins Substituted celluloses In Colowick S P & Kaplan N O (Eds) Methods in enzymology vol 5 Academic Press New York and London 1962 p 3–27

7 *Snyder F & Stephens N* A simplified spectrophotometric determination of ester groups in lipids Biochem biophys Acta 34 244–245 1959

8 *Sperry W M* Lipide analysis In Glick D (Ed) Methods of biochemical analysis vol 2 Interscience Publishers New York 1955 p 83–111

9 *Winzler R J* Determination of serum glycoproteins In Glick D (Ed) Methods of biochemical analysis vol 2 Interscience Publishers New York 1955 p 279–311

Acta path microbiol scand 77 457–464 1969

Laboratory of Oral Microbiology Department of Microbiology The Gade Institute and Institute of Periodontology Schools of Medicine and Dentistry University of Bergen Norway

IMMUNOCHEMICAL STUDIES OF ORAL FUSOBACTERIA

4 *Some Chemical Properties of a Group Reactive Precipitinogen*

By

TORE KRISTOFFERSEN

Received 23 iv 69

In a previous communication the isolation of a group reactive precipitinogen from a strain of *Fusobacterium* was described (15) The product provisionally named Precipitinogen 2 was found to be homogeneous in paper electrophoresis gel filtration agar precipitation and immune electrophoresis experiments The material was very unstable in solution and was readily denatured during purification procedures particularly during the final concentration and desalting processes

The present paper deals with some chemical properties of the purified Precipitinogen 2

MATERIALS

Preparation no 1 The batch of Precipitinogen 2 for which the purification procedure was described in detail in (15) The lyophilized material was white light and gave clear solutions in aqueous media at neutral pH

Preparations nos 2 and 3 Precipitinogen 2 which had been prepared exactly as described in (15) except that the final concentration and desalting was achieved through ultrafiltration and dialysis This gave a considerably higher yield of freeze dried material although most of the serological activity was lost in these final steps The freeze dried product was only slightly soluble in aqueous media even at strongly alkaline pH The specific activity (15) of these preparations prior to the final concentration and desalting procedures was the same as with Preparation no 1

The source of all antigen preparations was strain F1

Rabbit antiserum against whole bacteria strain F1 was prepared as reported earlier (13) The undiluted antiserum was mixed with an equal volume of 0 2M 2 mercaptoethanol After one hour at room temperature the mixture was dialyzed for 4 hours against 0 6 M iod acetamide and then against buffered saline over night

---

The quantitative amino acid analysis was performed by Mr *Jens Roe* Department of clinical Biochemistry University of Bergen School of Medicine The thin layer chromatography for lipids was done by Mrs *Inger Grundt* of the same department The author is indebted to these persons and to the head of the department Professor Karl Closs M D Ph D for their help and advice

These investigations have been supported by grants from *L Meltzers Høyskolefond* and from the *Norwegian Research Council for Science and the Humanities*

DISCUSSION

The qualitative analyses of the three preparations showed very good agreement The components identified by the paper chromatographic methods used were 14 amino acids the sugars glucose and xylose and a small lipid component

Experiments reported in a previous communication (13) showed that the precipitating ability of Precipitinogen 2 was destroyed by proteolytic enzymes and by periodate oxidation indicating the presence of both protein and carbohydrate The results of paper electrophoresis experiments with and without borate in the electrophoresis buffers strongly suggest that protein and carbohydrate are linked together (10) This assumption was corroborated by the demonstration of both glucose and xylose in a specific immune precipitate Rabbit IgG contains mannose and galactose roughly in the ratio 2 1 and fucose but neither glucose nor xylose (6) Since a protein component was found to be essential for the precipitating ability of Precipitinogen 2 (13) the glucose and xylose found in the immune precipitate most likely are linked to this protein It appears unlikely that the glucose and xylose found in the immune precipitate should be parts of a contaminating precipitating antigen No indication of the presence of such contaminants has been found (15)

The nitrogen hexose pentose and fatty acid esters found in preparation no 2 account for some 95 per cent of the material without correction for water uptake during hydrolysis The analysis of preparation no 3 gave less satisfactory results Both nitrogen and sugar values were lower than in preparation no 2 whereas the contents of lipid and phosphorus were somewhat higher

Variations in the lipid content in different batches of purified antigens form Gram negative bacteria are frequently reported (12 19) The somewhat divergent results of the quantitative analyses of the two preparations may partially be due to variations in the lipid contents not detectable by the hydroxamic acid assay as applied in the present study The small amounts of material available for analysis have prevented further studies of the lipid component Pilot experiments have indicated the presence of phospholipids along with other lipid classes however The higher phosphorus content in preparation no 3 might be due to a higher content of phospholipids

The Winzler orcinol assay gave very reproducible results and is believed to give reliable estimates of neutral sugars in glycoproteins even in the presence of small amounts of tryptophan (17) Other substances known to interfere in this assay were not detected

It is difficult to ascertain which of the two reactions for pentoses produced the most accurate results Since no heptose was found in Precipitinogen 2 and since non carbohydrates are considered to in

terfere less in the cysteine sulphuric acid reaction (11, 4), the values from the latter assay have been listed in Table 1.

In addition to the amino acids identified on the paper chromatograms, the automatic amino acid analyzer established the presence of phenylalanine, histidine and trace amounts of methionine and separated isoleucine and leucine. Several amino acids are liable to be decomposed during acid hydrolysis. Tryptophan is very readily destroyed but losses may be expected also for cysteine, cystine and tyrosine, especially in the presence of carbohydrate (5). Since the preparation used for amino acid analysis gave a completely clear solution in 0.1N NaOH, tryptophan could be estimated by direct spectrophotometry. The absence in chromatograms of all hydrolysates of cystine, cysteine and tyrosine did not encourage special efforts to possibly identify and quantitate these amino acids.

No trace of diaminopimelic acid was found in any of the three preparations of Precipitinogen 2. This observation may be of some interest in view of the significance and almost ubiquitous occurrence of this amino acid in Gram-negative bacteria (20). Studies by *Baird-Parker* (2) as well as in our laboratory (14) have shown that diaminopimelic acid is present in *Fusobacterium* including strain F1.

In the Rondle and Morgan assay for amino sugars a faint red colour was produced by both preparations analyzed. Since no amino sugars were detected on paper or column chromatography of acid hydrolysates, this colour was regarded as non-specific. Mixtures of amino acids, particularly lysine, with neutral sugars are known to give red colours in the Elson-Morgan assay (8).

In view of the strong indications of a linkage between carbohydrate and protein in Precipitinogen 2, the failure to detect amino sugars is a matter of considerable interest. In most known instances a carbohydrate-protein linkage involves an amino sugar, usually an acetyl-D-hexosamine (18). Few exceptions are known. However, there is strong evidence that other types of linkages than those involving amino sugars are possible. Thus the findings of *Lindahl & Rodén* (16) indicate that a glycosidic linkage between xylose and the hydroxyl group of serine constitutes the carbohydrate-peptide linkage in heparin. A similar linkage may be present in chondroitin 4-sulphate (9). Serine and xylose were invariably found in our preparations. However, no attempt has been made to elucidate the nature of the binding of carbohydrate to protein in Precipitinogen 2.

SUMMARY

Some chemical properties of a group reactive precipitinogen from the *Fusobacterium* strain F1 have been investigated. One preparation contained approximately 85 per cent protein including 15 amino acids, a carbohydrate component consisting of 9.1 per cent glucose and 0.7 per

DISCUSSION

The qualitative analyses of the three preparations showed very good agreement. The components identified by the paper chromatographic methods used were 14 amino acids, the sugars glucose and xylose and a small lipid component.

Experiments reported in a previous communication (13) showed that the precipitating ability of Precipitinogen 2 was destroyed by proteolytic enzymes and by periodate oxidation, indicating the presence of both protein and carbohydrate. The results of paper electrophoresis experiments with and without borate in the electrophoresis buffers strongly suggest that protein and carbohydrate are linked together (10). This assumption was corroborated by the demonstration of both glucose and xylose in a specific immune precipitate. Rabbit IgG contains mannose and galactose roughly in the ratio 2 : 1 and fucose, but neither glucose nor xylose (6). Since a protein component was found to be essential for the precipitating ability of Precipitinogen 2 (13), the glucose and xylose found in the immune precipitate most likely are linked to this protein. It appears unlikely that the glucose and xylose found in the immune precipitate should be parts of a contaminating precipitating antigen. No indication of the presence of such contaminants has been found (15).

The nitrogen, hexose, pentose and fatty acid esters found in preparation no. 2 account for some 95 per cent of the material without correction for water uptake during hydrolysis. The analysis of preparation no. 3 gave less satisfactory results. Both nitrogen and sugar values were lower than in preparation no. 2, whereas the contents of lipid and phosphorus were somewhat higher.

Variations in the lipid content in different batches of purified antigens form Gram negative bacteria are frequently reported (12, 19). The somewhat divergent results of the quantitative analyses of the two preparations may partially be due to variations in the lipid contents not detectable by the hydroxamic acid assay as applied in the present study. The small amounts of material available for analysis have prevented further studies of the lipid component. Pilot experiments have indicated the presence of phospholipids along with other lipid classes, however. The higher phosphorus content in preparation no. 3 might be due to a higher content of phospholipids.

The Winzler orcinol assay gave very reproducible results and is believed to give reliable estimates of neutral sugars in glycoproteins even in the presence of small amounts of tryptophan (17). Other substances known to interfere in this assay were not detected.

It is difficult to ascertain which of the two reactions for pentoses produced the most accurate results. Since no heptose was found in Precipitinogen 2 and since non carbohydrates are considered to in

terfere less in the cysteine sulphuric acid reaction (11 4) the values from the latter assay have been listed in Table 1

In addition to the amino acids identified on the paper chromatograms the automatic amino acid analyzer established the presence of phenylalanine histidine and trace amounts of methionine and separated *iso*leucine and leucine Several amino acids are liable to be decomposed during acid hydrolysis Tryptophan is very readily destroyed but losses may be expected also for cysteine cystine and tyrosine especially in the presence of carbohydrate (5) Since the preparation used for amino acid analysis gave a completely clear solution in 0 1N NaOH tryptophan could be estimated by direct spectrophotometry The absence in chromatograms of all hydrolysates of cystine cysteine and tyrosine did not encourage special efforts to possibly identify and quantitate these amino acids

No trace of diaminopimelic acid was found in any of the three preparations of Precipitinogen 2 This observation may be of some interest in view of the significance and almost ubiquitous occurrence of this amino acid in Gram negative bacteria (20) Studies by *Baird Parker* (2) as well as in our laboratory (11) have shown that diaminopimelic acid is present in *Fusobacterium* including strain F1

In the Rondle and Morgan assay for amino sugars a faint red colour was produced by both preparations analyzed Since no amino sugars were detected on paper or column chromatography of acid hydrolysates this colour was regarded as non specific Mixtures of amino acids particularly lysine with neutral sugars are known to give red colours in the Elson Morgan assay (8)

In view of the strong indications of a linkage between carbohydrate and protein in Precipitinogen 2 the failure to detect amino sugars is a matter of considerable interest In most known instances a carbohydrate protein linkage involves an amino sugar usually an acetyl D hexosamine (18) Few exceptions are known However there is strong evidence that other types of linkages than those involving amino sugars are possible Thus the findings of *Lindahl & Roden* (16) indicate that a glycosidic linkage between xylose and the hydroxyl group of serine constitues the carbohydrate peptide linkage in heparin A similar linkage may be present in chondroitin-4 sulphate (9) Serine and xylose were invariably found in our preparations However no attempt has been made to elucidate the nature of the binding of carbohydrate to protein in Precipitinogen 2

SUMMARY

Some chemical properties of a group reactive precipitinogen from the *Fusobacterium* strain F1 have been investigated One preparation contained approximately 85 per cent protein including 15 amino acids a carbohydrate component consisting of 9 1 per cent glucose and 0 7 per

cent xylose and a small amount of lipid Other preparations contained the same components There was however considerable variations in the relative quantities of the components between different preparations

Though no amino sugars were found the carbohydrate and protein appeared to be linked together The nature of the binding is not known

REFERENCES

1 *Augustinsson K B* Eksperimentell biokemi Scandinavian university books Svenska Bokforlaget Stockholm 1966

2 *Baird Parker A C* The classification of fusobacteria from the human mouth J gen Microbiol 22 458–469 1960

3 *Beaven G H* & *Holiday E R* Ultraviolet absorption spectra of proteins and amino acids Advanc Protein Chem 7 319–386 1952

4 *Dische Z* New color reactions for determination of sugars in polysaccharides In Glick D (Ed) Methods of biochemical analysis vol 2 Interscience Publishers Inc New York 1955 p 313–358

5 *Eastoe J E* Amino acid analysis of glycoproteins In Gottschalk A (Ed) Glycoproteins Elsevier Publishing Co Amsterdam 1966 p 112–150

6 *Fleischman J B Porter R R* & *Press E M* The arrangement of the peptide chains γ globulin Bioch m J 88 220–228 1963

7 *Freeman C P* & *West D* Complete separation of lipid classes on a single thin layer plate J Lipid Res 7 324–327 1966

8 *Gardell S* Determination of hexosamines In Glick D (Ed) Methods of biochemical analysis vol 6 Interscience Publishers Inc New York 1958 p 289–317

9 *Gregory J D Laurent T C* & *Rodén L* Enzymatic degradation of chondromucoprotein J biol Chem 239 3 12–3320 1964

10 *Haurowitz F* The chemistry and function of proteins Academic Press New York and London II ed 1963

11 *Kabat E A* & *Mayer M M* Experimental immunochemistry Thomas Springfield II ed 1961

12 *Kasai N* Chemical studies on the lipid component of endotoxin with special emphasis on its relation to biological activities Ann N Y Acad Sci *133* 486–507 1966

13 *Kristoffersen T* Immunochemical studies of oral fusobacteria I Major precipitinogens Acta path microbiol scand 77 235–246 1969

14 *Kristoffersen T* Immunochemical studies of oral fusobacteria II Some properties of undigested cell wall preparations Acta path microbiol scand 77 247–257 1969

15 *Kristoffersen T* Immunochemical studies of oral fusobacteria III Purification of a group reactive precipitinogen Acta path microbiol scand 77 431–440 1969

16 *Lindahl U* & *Rodén L* The linkage of heparin to protein Biochem biophys Res Commun *17* 254–259 1964

17 *Neuberger A* & *Marshall R D* Methods for the qualitative and quantitative analysis of the component sugars In Gottschalk A (Ed) Glycoproteins Elsevier Publishing Co Amsterdam 1966 p 190–234

18 *Neuberger A Gottschalk A* & *Marshall R D* Carbohydrate peptide linkages in glycoproteins and methods for their elucidation In Gottschalk A (Ed) Glycoproteins Elsevier Publishing Co Amsterdam 1966 p 273–295

19 *Ribi E Anacker R L Fukushi K Haskins W T Landy M* & *Milner K C* Relationship of chemical composition to biological activity In Landy M & Braun W (Eds) Bacterial endotoxins Rutgers University Press New Brunswick 1964 p 16–28

20 *Salton M R J* The bacterial cell wall Elsevier Publishing Co Amsterdam 1964

Acta path microbiol scand 77 465–476 1969

The Department of Medical Microbiology (Head Professor R. Grubb)
University of Lund Lund Sweden

PRODUCTION OF ANTIBIOTICS BY *EPIDERMOPHYTON FLOCCOSUM*

4 *The Antibacterial Activity of the 'Epidermophyton factor (EPF) Compared with that of some Steroid Antibiotics*

By

ALF WALLERSTROM

Received 13 v 69

Previous publications (*Wallerstrom* 1967 1968 1969) were concerned with the course of formation the antibacterial spectrum and some other properties of an antibiotic produced by a skin pathogenic fungus *Epidermophyton floccosum* (the Epidermophyton factor below called EPF) The antibiotic proved to have a characteristic antibacterial spectrum differentiating between *inter alia* corynebacteria and *Listeria*, and between staphylococci and streptococci In this and other respects EPF resembled fucidin and in the first survey (*Wallerstrom* 1967) it was found that EPF resistant staphylococcal strains were resistant also to the latter antibiotic

Fucidin belongs to a group of steroid antibiotics chemically characterized as unsaturated monobasic carboxylic acids with a protolanostane skeleton probably all related to the sterol triterpene family (*Stewart* 1964) Antibiotics of this group have similar antibacterial spectra and cross resistance has been found between several of them (*Burton & Abraham* 1951) *Barber & Waterworth* 1962)

This paper concerns a comparison of the antibacterial spectrum and some other properties of EPF and the above mentioned group of steroid antibiotics As representatives of this group those that have been most thoroughly investigated were selected namely helvolic acid (fumigacin) cephalosporin P_1 and fucidin

MATERIAL AND METHODS

Antibiotics Samples of EPF were prepared from ether extracts of filtrates from submerged cultures of *Epidermophyton floccosum* as described earlier (*Wallerstrom* 1969) In order to eliminate penicillin which may be produced by *Epidermophyton* strains Difco Bactopenase was added to the filtrates before extraction Samples of steroid antibiotics were kindly supplied by Professor E P Abraham Oxford England and by Dr W O Godtfredsen Løvens Kemiske Fabrik Ballerup Denmark

Bacterial strains In studies on the antibacterial spectrum of EPF and steroid antibiotics 27 strains belonging to 12 different bacterial species (Table 1) were

used Most strains had recently been isolated from routine laboratory specimens as for β streptococci other than group A *Corynebacterium diphtheriae* and *Listeria monocytogenes* older or freeze dried strains were used *Neisseria pharyngis* and *N catarrhalis* were defined as described earlier (*Wallerström* 1967) —In addition a number of strains of micrococci and staphylococci that had been collected from skin lesions due to *F floccosum* or from healthy or yeast infected individuals in a previous study (*Wallerstrom* 1968) were studied for their susceptibility to EPF and fucidin only a few strains of EPF resistant staphylococci found in nose and throat swabs (*Wallerström* 1967) were tested in the same way

Media In most tests for antibiotic activity on solid media Oxoid Diagnostic Sensitivity Test Agar (DST agar) was used Streptococci pneumococci *Neisseria pharyngis* and *N catarrhalis* were tested on plates containing Oxoid Blood Agar Base No 2 and 4 per cent horse blood *N meningitidis* on hematin agar prepared from the same base and 7 per cent heated horse blood *N gonorrhoeae* was tested on a special medium containing horse blood and ascitic fluid (*Reyn et al* 1963) The liquid medium used in most experiments was Oxoid Nutrient Broth No 2 and in some experiments concerning increase of resistance of staphylococci and coryne bacteria to EPF or fucidin Oxoid Tryptone Soya Broth

Gradient plates 15 ml of DST agar containing 40 U/ml of EPF was poured into a slanting petri dish with a diameter of 85 mm and allowed to solidify An upper layer of 15 ml of DST agar containing no antibiotic was added the dish now being horizontal The maximal thickness of each layer was 6 mm The plates were used for experiments as soon as the top layer had solidified

Gel filtration Ether extracts of EPF used *inter alia* in experiments concerning antibacterial spectrum and comparison with steroid antibiotics were partially purified by gel filtration A Sephadex G 10 column K 15/90 measuring 15 × 7?0 mm (bed volume approximately 125 ml) was used with distilled water as eluent The EPF extracts were applied in volumes of 5–15 ml The eluent was run at a rate of 15 ml per h and collected in 10 minute fractions Fractions containing EPF as identified by their antibacterial activity in the agar diffusion test were pooled and concentrated by pervaporation at room temperature

In vitro increase of resistance of bacteria to EPF and fucidin Broth cultures of strains of *Staph aureus* one of which was the standard test strain used in a previous investigation (*Wallerstrom* 1969) were started from single colonies and used for inoculation of tubes with 2 ml broth containing 10 U/ml of EPF The first inoculum used was heavy 0 5 ml of a 20 h culture The bacteria were subcultured still once or twice on media with this concentration of EPF tubes containing ?0 U/ml of EPF were then inoculated The inoculum of the subcultures varied between 0 04–0 15 ml depending on the density of the growth the incubation time was ?0 h but when growth was poor the incubation time was extended to 48–7? h An ana logous procedure was used for fucidin the amount of fucidin in the medium was successively increased from 2 to 100 mcg per ml in 3 steps

3 strains of *Corynebact diphtheriae* first had to be cultivated on media with a low concentration of EPF 2 U/ml subpassages were then made on media in which the concentration of EPF was increased in several steps to a final value of 15 U/ml —Two strains of *Corynebact spp* on the other side could be cultivated directly on media containing 15 U/ml of EPF

The susceptibility to antibiotics was tested by the agar diffusion test The supply of helvolic acid and cephalosporin P_1 was sparse therefore in experiments where the susceptibility of the bacteria to EPF was compared with that of steroid anti biotics paper discs were prepared. The discs had a diameter of 6 mm (manuf Schleicher & Schuell) and were imbibed with 0 01–0 04 ml of the antibiotic solution and dried As the antibiotics were only slightly soluble in water but soluble in acetone the latter solvent was used The amount of antibiotic in the discs was 0 35 –2 mcg of fucidin 0 75–7 5 mcg of cephalosporin P_1 1 75–14 mcg of helvolic acid and 0 5–15 U of EPF (discs of four different strengths were made for each antibiotic) Discs giving identical or near identical zones with the standard strain of *Staph aureus* were chosen for the comparisons but each bacterial strain was tested with at least two different discs for each antibiotic The activity of the discs was checked every day against the standard strain of *Staph aureus*

A larger number of strains of staphylococci and micrococci were tested for their susceptibility to EPF and fucidin The tests were performed with agar cup techni que on flooded plates (*Wallerstrom* 1967) a sample of EPF containing 1?0 U per

ml was used The susceptibility to fucidin was tested with commercial discs containing 50 mcg of the antibiotic Some strains were tested also for their susceptibility to other antibiotics with discs from the same manufacturer (AB Biodisk, Stockholm 60 Sweden)

Testing of bactericidal/bacteriostatic activity A series of tubes containing different amounts of EPF (0 2-200 U/ml) were inoculated with approximately 10^3 bacteria per ml In this and the following experiments the inoculum consisted of staphylococci from a 20 h broth culture which had been started from a single colony the bacteria had been washed and resuspended in phosphate buffer pH 7 2 and the suspension treated for 5 minut s in a MSE Mullard ultrasonic disintegrator (frequency about 20 kilocycl s/sec) Viable counts showed that the treatment produced an approximately 30 per cent increase of the numl er of colonies growing from the suspension Treatment for longer periods did not produce any further increase in the number After incubation at 37 C for 24 h the tubes were tested for viable organisms by spreading a standard inoculum over the surface of two agar plates The plates were afterwards incubate l at 37 C for 24 h. The lowest concentration at which no increase of turbidity occurred in the tubes was considered the bacteriostati endpoint (minimal inhibitory concentration m i c) The lowest concentration at which no viable organisms were found on plating was considered the bactericidal end point (minimal bacterici lal concentration m b c) In some experiments the incubation of the tubes was prolonged and viable counts were made after 2 3 and 4 days

In another experiment two series of tubes with 2 ml nutrient broth containing 5 20 and 40 U/ml of EPF respectively were inoculated with approximately 1 5 × 10^1 bacteria/ml from a 20 h broth culture of *Staph aureus* and incubated at 37 C. Surface viable counts were made after incubation for 2 4 and 24 h Each count was made by diluting samples 1 10 and 1 100 and spreading 0 1 ml of each on agar plates in duplicate

The number of EPF resistant cells in a population of staphylococci was te ted by inoculating EPF containing solid media with predetermined numbers of *Staph aureus* from a 20 h broth culture. The inocula were prepared by suspending the bacteria in a volume of phosphate buffer corresponding to that of the original broth culture or in one tenth of this volume Each plate received 0 1 or 0 5 ml of the bacterial suspension The surface of the plate was about 60 cm² *The number of bacteria in each suspension* was checked by diluting it 1 10^5 1 10^6 and 1 10^7 and spreading 0 1 ml of each dilution on antibioti free agar plates The number of EPF resistant mutants was estimated by counting and comparing the number of colonies that had grown on agar plates with and without EPF after incubation at 37 C for 48 h

RESULTS

The Antibacterial Spectrum of Steroid Antibiotics Compared with That of EPF

A number of bacterial strains were tested by the agar diffusion test for their susceptibility to EPF helvolic acid cephalosporin P_1 and fucidin The results of the tests are given in Table I

It is clear from the table that the antibacterial spectrum of EPF resembled that of cephalosporin P_1 and fucidin Helvolic acid was found to differ from the other substances in several respects being relatively more active against β hemolytic streptococci pneumococci and Neisseriae with the possible exception of N gonorrhoeae

The quantity of fucidin in the discs necessary to achieve a 24–25 mm inhibition zone with staphylococci was about half that of cephalosporin P_1 and one tenth of that of helvolic acid

In an earlier investigation (Wallerstrom 1968) a selected material of 390 strains of staphylococci and micrococci were tested for their

TABLE 1

Antibacterial Spectra of Some Steroid Antibiotics and of EPF Inhibition Zones (in mm) in the Agar Diffusion Test

	No of strains	Helvol acid 7 mcg disc.	Cephalosp P_1 1 5 mcg disc	Fucidin 0 7 mcg disc	EPF 5 U disc.
Staphylococcus aureus					
(EPF susc test strain)	1	25	24	24	24
Staphylococcus aureus					
(EPF resistant strains)	4	0	0	0	0
α streptococci	2	0	0	0	0
β streptococci group A	1	23	0	0	0
β streptococci group A	2		±	0	0
β streptococci group H	1	21	0	0	0
β streptococci group L	1	25	0	0	0
Enterococci	1	±	0	0	±
Pneumococci	1	18	0	0	0
Corynebact dipht gravis	1			27	27
Corynebact dipht intermed	1			25	25
Corynebact dipht mitis	1	31	27	23	23
Corynebacterium spp					
strain 1	1	13	11 5	12 5	10
strain 2	1		23	16	15
strain 3	1	30		24	18
Listeria monocytogenes	1	±	0	0	0
Neisseria pharyngis	1	19 5	0	0	0
Neisseria catarrhalis	1	14 5	±	±	±
Neisseria meningitidis					
strain 1	1	18	9 5	10 5	10
strain 2	1	24		15	14
Neisseria gonorrhoeae	2	0–±	0	0	0

susceptibility to EPF and penicillin These strains were now tested against fucidin All EPF sensitive strains (301) proved sensitive to fucidin and the EPF resistant strains (89) were resistant to fucidin Strains whose growth was inhibited in large zones by fucidin (more than 35 mm in diameter with commercial discs containing 50 mcg of the antibiotic) also showed large inhibition zones when tested with EPF (mean value of zone diameter 32 7 mm)

In Vitro Increase of Resistance of Bacterial Strains to EPF and to Fucidin

In experiments with 9 originally EPF sensitive strains of *Staph aureus* the strains were found to grow well in the presence of successive[ly] increasing concentrations of EPF when the inoculum used w[as] [m]ore than 10^7 bacteria per ml) After 4–7 subcultures in [u]p to 20 U/ml of EPF no zones occurred in the agar In another series of experiments the resistance of [fuc]idin was increased in the same way by sub

culturing the strains on media containing up to 100 mcg per ml of fucidin

Three strains of *Corynebact diphteriae* and two strains of *Corynebacterium spp* were propagated in the way described above on media containing successively increasing amounts of EPF eventually 15 U/ml After having been subcultured twice on media with the last mentioned concentration no inhibition zones occurred with the strains in the agar plate diffusion test with FPF whereas the sensitive parent strains showed zones which were 23–27 mm in diameter

In another experiment staphylococcal strains were cultured over night in liquid media containing a low concentration of EPF (1–2 U/ml) The inhibiton zones in subsequent agar diffusion tests diminished and a number of small colonies appeared in the zones Such colonies were tested for their susceptibility to FPF in the tube dilution test They were found to have a m i c of 225 U/ml whereas the m i c of the parent strains was 0 2–0 5 U/ml The corresponding zones in the agar diffusion test had a diameter of 15 and 31 mm respectively

Experiments were made where gradient plates containing 40 U/ml of FPF in the bottom layer were flooded with staphylococci from two broth cultures that had been started from single colonies Each plate received approximately 10^r bacteria After incubation for 48 h 5 colonies which had grown on different gradient levels of each plate were isolated and tested for their susceptibility to FPF by the agar diffusion test The inhibition zones ranged between 17 and 20 mm in diameter and were thus of the same order of magnitude The inhibition zones of the parent strains were 31 mm

Demonstration of Cross Resistance between EPF and Steroid Antibiotics

It was found that staphylococci which were able to grow on media containing FPF had a decreased susceptibility to fucidin and *vice versa* (Table 2)

Cross resistance has been demonstrated between fucidin and cephalosporin P_1 (*Barber & Waterworth* 1962) and between the latter antibiotic and helvolic acid (*Burton & Abraham* 1951) For this reason some of the staphylococcal strains that had increased their resistance to FPF and fucidin were tested for their susceptibility to cephalosporin P_1 and helvolic acid as were their non resistant parent strain Cross resistance was found between all of the three steroid antibiotics and between these antibiotics and FPF (Table 3)

The susceptibility of the bacteria to tetracycline was tested since some EPF resistant strains of staphylococci encountered in an earlier investigation (*Wallerstrom* 1967) were resistant also to this antibiotic In the present study the development of resistance to FPF and steroid antibiotics was not found to influence on the susceptibility of the bacteria to tetracycline

TABLE 1

Antibacterial Spectra of Some Steroid Antibiotics and of EPF Inhibition Zones (in mm) in the Agar Diffusion Test

	No of strains	Helvol. acid 7 mcg disc.	Cephalosp P_1 1 5 mcg disc	Fucidin 0 7 mcg disc.	EPF 5 U disc.
Staphylococcus aureus					
(EPF susc test strain)	1	25	24	24	24
Staphylococcus aureus					
(EPF resistant strains)	4	0	0	0	0
α streptococci	2	0	0	0	0
β streptococci group A	1	23	0	0	0
β streptococci group A	2		±	0	0
β streptococci group H	1	21	0	0	0
β streptococci group L	1	25	0	0	0
Enterococci	1	±	0	0	±
Pneumococci	1	18	0	0	0
Corynebact diphl gravis	1			27	27
Corynebact diphl intermed	1			25	25
Corynebact diphl mitis	1	31	27	23	23
Corynebacterium spp					
strain 1	1	13	11 5	12 5	10
strain 2	1		23	16	15
strain 3	1	30		24	18
Listeria monocytogenes	1	±	0	0	0
Neisseria pharyngis	1	19 5	0	0	0
Neisseria catarrhalis	1	14 5	±	±	±
Neisseria meningitidis					
strain 1	1	18	9 5	10 5	10
strain 2	1	24		15	14
Neisseria gonorrhoeae	2	0–±	0	0	0

susceptibility to EPF and penicillin These strains were now tested against fucidin All EPF sensitive strains (301) proved sensitive to fucidin and the EPF resistant strains (89) were resistant to fucidin Strains whose growth was inhibited in large zones by fucidin (more than 35 mm in diameter with commercial discs containing 50 mcg of the antibiotic) also showed large inhibition zones when tested with EPF (mean value of zone diameter 32 7 mm)

In Vitro Increase of Resistance of Bacterial Strains to EPF and to Fucidin

In experiments with 9 originally EPF sensitive strains of *Staph aureus* the strains were found to grow well in the presence of successively increasing concentrations of EPF when the inoculum used was large (more than 10^7 bacteria per ml) After 4–7 subcultures in media containing up to 20 U/ml of EPF no zones occurred in the agar plate diffusion test In another series of experiments the resistance of the staphylococci to fucidin was increased in the same way by sub

TABLE 3

Effect of Culturing Staphylococci in Media Containing EPF or Fucidin Increase in Resistance to EPF and Steroid Antibiotics Agar Plate Diffusion Test

Staph strain labelled	Original zone diameter (in mm)					Zone diameter after growth in media containing EPF (in mm)					Zone diameter after growth in media containing fucidin (in mm)				
	Helvolic acid	Cephalosporin P_1	Fucidin	EPF	Tetracycl (control)	Helvolic acid	Cephalosporin P_1	Fucidin	EPF	Tetracycl (control)	Helvolic acid	Cephalosporin P_1	Fucidin	EPF	Tetracycl (control)
I	21	22	26	22	31	0	0	13	(+)	33	(+)	0	0	0	30
II	20	23	26	23	13	0	0	0	0	15	0	0	0	0	15
III	20	22		22	29	0	12	12	0	30	0	0	0	0	30
IV	21	22	25	20	28	0	0	0	0	29	0	0	0	0	27 5
V	22	21 5	25	22	29	0	0	0	0	30	0	0	0	0	28
VI	22	5	25	23	27 5	0	0	0	0	30	0	0	0	0	28

TABLE 2

Effect of Culturing Staphylococci in Media Containing EPF or Fucidin. Mutual Increase of Resistance to Both Antibiotics. Agar Plate Diffusion Test

Staph strain labelled	Original zone diameter (in mm)		Zone diameter after growth in media containing EPF (in mm)		Zone diameter after growth in media containing fucidin (in mm)	
	EPF 120 U/ml in cup	Fucidin 50 mcg disc	EPF 120 U/ml in cup	Fucidin 50 mcg disc	EPF 120 U/ml in cup	Fucidin 50 mcg disc
I	24	35	0	24	0	17
II	25	35	0	17	0	21
III	25	34	0	21	0	0
IV	24	33	0	13	0	10
V	25	35	0	20	0	±
VI	27	36	0	11	0	15
VII	29	39	0	11	0	13
VIII	25	35	0	19	0	20
IX	23	34	0	26	0	13

The Number of EPF Resistant Mutants in a Staphylococcal Population

Solid media containing EPF were inoculated with predetermined numbers of bacteria from 20 h broth cultures of *Staph. aureus*. It was found that suspensions with the same number of bacteria as the original broth culture (in three experiments 3.6 × 10^8, 4.2 × 10^8 and 2.8 × 10^8 bacteria per ml) in most cases gave rise to no growth or only scattered colonies. 0.1 ml of suspensions with the tenfold number of bacteria per ml resulted in growth of 29, 3.5 and 16.5 staphylococcal colonies. When tested in the agar diffusion test, all colonies were found to have a lowered susceptibility to EPF; the m.i.c. determined in 15 colonies from a 20 U/ml plate ranged from 3 to 24 U/ml of EPF. The number of resistant mutants in the staphylococcal populations was estimated from these results at 1 out of 10^7–10^9.

Mode of Action of EPF on Bacteria (Bactericidal/Bacteriostatic Activity)

The standard test strain of *Staph. aureus* was inoculated into a series of broth tubes containing 0.2–200 U/ml of EPF. The inoculum used was approximately 10^3 bacteria per ml. The m.i.c. of the strain was 0.5 U/ml and the m.b.c. 15 U/ml. When the incubation of the tubes was extented to 3 or 4 days, no viable bacteria were recovered from tubes containing more than 3.5 U/ml of EPF.

When broth containing 5, 20 or 40 U/ml of EPF was inoculated with approximately 1.5 × 10^4 bacteria per ml, the number of bacteria growing from the culture persisted at the level of 1–3 × 10^4 after 2 and 6 h

TABLE 3

Effect of Culturing Staphylococci in Media Containing EPF or Fucidin Increase in Resistance to EPF and Steroid Antibiotics Agar Plate Diffusion Test

Staph strain labelled	Original zone diameter (in mm)					Zone diameter after growth in media containing EPF (in mm)					Zone diameter after growth in media containing fucidin (in mm)				
	Helvolic acid	Cephalosporin P_1	Fucidin	EPF	Tetracycl (control)	Helvolic acid	Cephalosporin P_1	Fucidin	EPF	Tetracycl (control)	Helvolic acid	Cephalosporin P_1	Fucidin	EPF	Tetracycl (control)
I	21	22	26	22	31	0	0	13	(+)	33	(+)	0	0	0	30
II	20	23	26	23	13	0	0	0	0	15	0	0	0	0	15
III	0	22	25	22	9	0	12	12	0	30	0	0	0	0	30
IV	21	2	20	20	28	0	0	0	0	29	0	0	0	0	27 5
V	22	21 5	25	[illegible]	29	0	0	0	0	30	0	0	0	0	28
VI	22	25	5	23	27 5	0	0	0	0	30	0	0	0	0	28

against pneumococci and β hemolytic streptococci (but not against α hemolytic streptococci) judging from the antibacterial spectra EPF is not identical with helvolic acid Chemical studies are in progress to ascertain whether EPF is identical with any of the other two antibiotics

Several derivatives of helvolic acid cephalosporin P_1 and fusidic acid with antibiotic activity have been produced in laboratories some of these derivatives have also been found as naturally occuring antibiotics produced by fungi With one exception (hydrogenation of the isolated double bond in the side chain of fusidic acid and cephalosporin P_1) all modifications of the molecular structure of these antibiotics hitherto reported have resulted in compounds with lower activity against the test bacteria (*Burton et al* 1955) *Godtfredsen et al* 1965) *Okuda et al* 1966) *Janssen & Vanderhaeghe* 1967)

Beside the above mentioned antibiotics and their derivatives other compounds exist which have a steroid skeleton and possess antibiotic properties polyporenic acid A and C eburicoic acid and viridin The two first mentioned substances have antibacterial activity but their antibacterial spectra are different from that of EPF polyporenic acid A is about 50 times more active against staphylococci than against *Clostridium* spp (*Efimenko et al* 1961) whereas EPF is about equally active against both (*Wallerstrom* 1967) Eburicoic acid and viridin are antifungal antibiotics (*Harvey et al* 1967) — It is noteworthy in this connection that even some animal steroids possess antimicrobial properties deoxycorticosterone for instance in a concentration of 80–250 mcg/ml is reported to inhibit the growth of dermatophytes yeasts and moulds as well as of gram positive bacteria gram negative bacteria with the exception of *Neisseria catarrhalis* are much less susceptible (*Lester & Hechter* 1958) *Maxwell et al* 1960) *Chattaway & Townsley* 1962)

The patterns of antibiotic activity of helvolic acid cephalosporin P_1 and fucidin found in this study were in agreement with literature data on these antibiotics (2 4 5 9 10 11 18 21) The activity of helvolic acid compared with that of cephalosporin P_1 was lower than what was reported by *Burton & Abraham* (1951) who found that cephalosporin P_1 was nearly twice as active against staphylococci as helvolic acid *Ritchie et al* (1951) on the other hand found cephalosporin P_1 to be 4 to 8 times more active than helvolic acid against the same bacterial species The results in the present study are closer to the latter value A possible explanation is that the sample of helvolic acid used in this study contained a small amount of 7 deacetyl helvolic acid as an impurity This substance is produced by fungi but may also be obtained on partial hydrolysis of helvolic acid it is also an antibiotic (synonym helvolinic acid) though its activity is only about one eight of that of helvolic acid (*Burton et al* 1956) According to *Okuda et al* (1966) its antibacterial spectrum is similar to that of helvolic acid

The question whether EPF is mainly bacteriostatic or mainly bac

tericidal was studied in two different ways When the antibiotic was allowed to act on a small amount of staphylococci for 24 h the minimal inhibitory concentration was 0 5 U/ml but the minimal bactericidal concentration was conciderably higher 15 U/ml (test technique I) However repeated viable counts in broth cultures of staphylococci during the first 24 h of exposure (test technique II) revealed in most instances a slow decrease in the number of organisms even at a con centration of 5 U/ml – As to steroid antibiotics *Godtfredsen et al* (1962 a) who used the latter technique and small inocula found that 10 mcg/ml of fusidic acid had a bactericidal effect on staphylococci *Garrod & Waterworth* (1956) who used the former technique found the same concentration of cephalosporin P_1 to have bactericidal ac tivity when the inoculum used was small (5 × 10^3 staphylococci per ml or less) when larger inocula were used surviving organisms were found *Chain et al* (1943) using heavy inocula found the m i c of helvolic acid on staphylococci to be 1 5 mcg/ml but the m b c was more than 25 mcg/ml

EPF resistant mutants of staphylococci were readily produced by culturing the strains on media containing EPF Mutants with a high degree of resistance were produced also on media with a very low con centration of EPF and in experiments with gradient plates colonies growing on different gradient levels of the plate showed zones of com parable sizes when tested against FPF in the agar diffusion test De velopment of resistance of this antibiotic is thus of the streptomycin or one step type rather than of the step wise (penicillin) type De velopment of resistance to fucidin is also claimed to be a one step mutation

The development of resistance to FPF by staphylococci did not change their susceptibility to a number of other antibiotics The find ing of resistance to tetracycline in some FPF resistant staphylococcal strains in an earlier investigation (*Wallerstrom* 1967) was thus in cidental – The conclusion by *Godtfredsen et al* (1962 b c) that fuci din resistant strains lose their ability to coagulate human plasma could not be verified in this study If it had been it would have fitted in with the observation that all EPF resistant staphylococcal strains found in skin lesions due to *Epidermophyton floccosum* were coagulase nega tive (*Wallerstrom* 1968) this has to be explained in some other way

EPF resembles helvolic acid cephalosporin P_1 and fucidin also in some other respects its antibacterial activity is reduced in the pre sence of serum and increased by acidification of the test media (*Wal lerstrom* 1969) Inhibition by serum and increased activity at lower pH is reported for all of the three steroid antibiotics mentioned

SUMMARY

The antibacterial spectrum of the *Epidermophyton* factor (EPF) which is produced by the skin pathogenic fungus *Epidermophyton floccosum* was compared with those of three steroid antibiotics helvolic acid cephalosporin P_1 and fucidin The spectra of the last two antibiotics were very similar whereas helvolic acid was relatively more active than EPF against *inter alia* β streptococci and pneumococci Staphylococci and corynebacteria readily developed resistance to EPF *in vitro* Strains of *Staphylococcus aureus* cultured on media containing EPF or fucidin were found to be resistant to each of these substances as well as to helvolic acid and cephalosporin P_1 In moderate concentrations EPF had bactericidal effect on staphylococci The frequency of resistant mutants in a staphylococcal population was 1 out of 10^7–10^9

REFERENCES

1 *Barber M & Waterworth P* Antibacterial activity *in vitro* of fucidin Lancet *I* 931–932 1962
2 *Burton H S & Abraham E P* Isolation of antibiotics from a species of *Cephalosporium* Cephalosporins P_1 P P P_4 and P_5 Biochem J *50* 168–174 1951
3 *Burton H S Abraham E P & Cardwell H M E* Cephalosporin P_1 and helvolic acid Biochem J *62* 171–176 1956
4 *Bulow Sørensen P* Fucidins (ZN 6) antibakterielle effekt *in vitro* Ugeskr Laeg *124* 745–769 1962
5 *Chain E Florey H W Jennings M A & Williams T I* Helvolic acid an antibiotic produced by *Aspergillus fumigatus* mut *helvola* Yuill Brit J exp Path *24* 108–119 1943
6 *Chattaway F W & Townsley J D* The effect of certain steroids upon the growth of *Trichophyton rubrum* J gen Microbiol *28* 437–441 1962
7 *Efimenko O M Mel nikova T A Zo ulya R A & Kostygov V M* Polyporenic acid A—an antibiotic isolated from the tinder fungus *Polyporus b tulinus* Antibiotiki *6* 215–220 1961
8 Garrod L P & Waterworth P M Behaviour *in vitro* of some new antistaphylococcal antibiotics Brit Med J 1956 II pp 61–65
9 *Godtfredsen W O Jahnsen S Lorck H Roholt K & Tybring L* Fusidic acid a new antibiotic Nature *193* 987 1962 a
10 *Godtfredsen W O Roholt K & Tybring L* Fucidin A new orally active antibioti Lancet I 928–931 1962 b
11 *Godtfredsen W O Lorck H Roholt K & Tybring L* Fucidin Et nyt antibiotikum til peroral behandling Ugeskr Laeg 124 715–719 1962 c
12 *Godtfredsen W O Albrethsen C Daehne W Tybring L & Vangedal S* Transformation of fusidic acid and the relationship between structure and antibacterial activity Antimicr Ag Chemother I 132–137 1965
13 *Godtfredsen W O Daehne W Tybring L & Vangedal S* Fusidic acid derivatives I Relationship between structure and antibacterial activity J Med Chem 9 15–22 1966
14 *Hartley C C Smith C J & Knight S G* Fusidic acid In *Gottlieb D & Shaw P D* Antibiotics Vol I Springer Verlag Berlin Heidelberg New York 1967 p 330
15 *Janssen G & Vanderhaeghe H* Modification of the side chain of fusidic acid (ramycin) J Med Chem *10* 205–208 1967
16 *Lester G & Hechter O* Effect of deoxycorticosterone on the growth of microorganisms J Bact 76 365 367 1958
17 *Maxwell F S McGuire J S & Tomkins C M* Antibiotic effect of steroids on *Sacharomyces fragilis* and the isolation of a resistant mutant J Bact 80 1–6 1960

tericidal was studied in two different ways When the antibiotic was allowed to act on a small amount of staphylococci for 24 h the minimal inhibitory concentration was 0 5 U/ml but the minimal bactericidal concentration was considerably higher 15 U/ml (test technique I) However repeated viable counts in broth cultures of staphylococci during the first 24 h of exposure (test technique II) revealed in most instances a slow decrease in the number of organisms even at a con centration of 5 U/ml – As to steroid antibiotics *Godtfredsen et al* (1962 a) who used the latter technique and small inocula found that 10 mcg/ml of fusidic acid had a bactericidal effect on staphylococci *Garrod & Waterworth* (1956) who used the former technique found the same concentration of cephalosporin P_1 to have bactericidal ac tivity when the inoculum used was small (5×10^5 staphylococci per ml or less) when larger inocula were used surviving organisms were found *Chain et al* (1943) using heavy inocula found the m i c of helvolic acid on staphylococci to be 1 5 mcg/ml but the m b c was more than 25 mcg/ml

EPF resistant mutants of staphylococci were readily produced by culturing the strains on media containing EPF Mutants with a high degree of resistance were produced also on media with a very low con centration of EPF and in experiments with gradient plates colonies growing on different gradient levels of the plate showed zones of com parable sizes when tested against EPF in the agar diffusion test De velopment of resistance of this antibiotic is thus of the streptomycin or one step type rather than of the step wise (penicillin) type De velopment of resistance to fucidin is also claimed to be a one step mutation

The development of resistance to EPF by staphylococci did not change their susceptibility to a number of other antibiotics The find ing of resistance to tetracycline in some EPF resistant staphylococcal strains in an earlier investigation (*Wallerstrom* 1967) was thus in cidental – The conclusion by *Godtfredsen et al* (1962 b c) that fuci din resistant strains lose their ability to coagulate human plasma could not be verified in this study If it had been it would have fitted in with the observation that all EPF resistant staphylococcal strains found in skin lesions due to *Epidermophyton floccosum* were coagulase nega tive (*Wallerstrom* 1968) this has to be explained in some other way

EPF resembles helvolic acid cephalosporin P_1 and fucidin also in some other respects its antibacterial activity is reduced in the pre sence of serum and increased by acidification of the test media (*Wal lerstrom* 1969) Inhibition by serum and increased activity at lower pH is reported for all of the three steroid antibiotics mentioned

Acta path microbiol scand 77 477–488 1969

Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt University of Oslo
Rikshospitalet Oslo Norway

INFLUENCE OF INHIBITORS OF DNA AND PROTEIN SYNTHESIS ON THE KINETICS OF DNA UPTAKE IN *NEISSERIA MENINGITIDIS* TRANSFORMATION

By

KAARE JYSSUM

Received 1 v 69

Kinetic measurements of the DNA uptake have been performed in most transformable species In *Streptococcus pneumoniae* linear rates for entry terminated by deoxyribonuclease have been found with both single and linked markers (15) There is a little lag before the first detected entry of each marker The addition of DNA to competent *B subtilis* cells is followed by an unmeasurably rapid irreversible attachement of DNA to the cells (8 18) Subsequently a period of approximately 1 minute ensues during which the potential transformants are sensitive to deoxyribonuclease The lag period which is independent of the single marker selected is considered to represent the time necessary for entry of a length of DNA which is long enough to participate in a recombination event The process of uptake of DNA in *Haemophilus influenzae* cells seems to be much more rapid than in pneumococcus and in *B subtilis* (30) But also in this microbe there seems to be a linear increase of transformants with time The uptake curve does not go through the origin but extrapolates to a value of about 2 5 sec when saturating concentrations of DNA have been used

In *Neisseria meningitidis* there seems to be a slower rate of DNA uptake in the cell than in the other systems more thoroughly examined and no lag has been observed before the first detected entry of a marker (5 20) The purpose of this investigation has been to study these differences in kinetics More specifically the following questions have been posed What is the metabolic situation during DNA uptake in competent cells of this species and what are the metabolic requirements for such uptake?

MATERIALS AND METHODS

Strains

The following mutants were obtained from the wild type strain MI of sero group B as previously described (14) MI 18 *his gly cp* MI 8 *his arg cp* MI 5 *his hom cp*

18 *Menzel A E Wintersteiner O & Hoogerheide J C* The isolation of gliotoxin and fumigacin from culture filtrates of *Aspergillus fumigatus* J Biol Chem 152 419–429 1944
19 *Okuda S Nakayama Y & Tsuda K* Microbial products I Helvolic acid and related compounds 1 7 deacetoxyhelvolic acid and helvolinic acid Chem Pharm Bull (Tokyo) *14* 436–441 1966
20 *Reyn A Weis Bentzon M & Ericsson H* Comparative investigation of the sensitivity of N gonorrhoeae to penicillin Acta path microbiol Scand *57* 235–255 1963
21 *Ritchie A C Smith N & Florey H W* Some biological properties of cephalosporin P_1 Brit J Pharmacol *6* 430–444 1951
22 *Stewart G T* Steroid antibiotics Pharmakotherapia (Munich) *2* 137–148 1964
23 *Wallerstrom A* Production of antibiotics by *Epidermophyton floccosum* I The antibiotic spectrum of crude filtrate Acta path microbiol Scand *71* 287–295 1967
24 *Wallerstrom A* Production of antibiotics by *Epidermophyton floccosum* II Microflora in *Epidermophyton* infected skin and its resistance to antibiotics produced by the fungus *ibid 74* 531–542 1968
25 *Wallerström A* Production of antibiotics by *Epidermophyton floccosum* III Course of formation and some properties of the Epidermophyton factor (EPF) *ibid* 75 435–446 1969

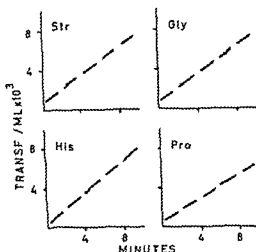


Fig 1

Linear accumulation of singly marked transformants in stirred mixtures of bacteria and DNA at 37 Calculated regression line for Str r is x = 1 44 y − 1 01 for Gly x = 1 46 y − 1 00 for His x = 1 28 y − 0 26 for Pro x = 1 73 y − 1 03

becomes likely that they extrapolate to a point before zero time i e to a time before the transforming DNA has actually been added From statistical analysis of covariation there appeared no objection against the assumption that the calculated straight lines represent the experimental values

Interference with the Linearity of the Uptake Curve

The kinetic curves of Fig 1 demonstrate the pattern observed when the recipient cells are in the early logarithmic phase of growth when DNA is added Manipulations which reduce the metabolic activity of the cells had a pronounced influence on the form of the curve When the metabolic activity was temporarily reduced by a cold shock before the addition of DNA a pronounced lag was obtained (Fig 2) during which a gradually increasing rate of appearance of transformants occurred

2 4 dinitrophenol is a potent inhibitor of oxidative phosphorylation with activity also on the metabolism of *N meningitidis* (13) Preincubation with 2 4 dinitrophenol had two effects on the transformation curve Fig 3 shows that a significant lag occurred before the appearance of the first transformants Furthermore the rate of appearance of transformants was reduced Further experiments showed that these effects were independent of any particular genetic trait among those tested but seemed to be characteristic of the *N meningitidis* transformation process in general when carried out under these conditions

Ml 48 *his cys cp* Ml 6 *his pro cp* Genetic competence is indicated by the symbol *cp* and incompetence which does not revert to competence by *cp* (1?) The *str* marker was a single step high level resistant mutant (11)

Media

Blood agar or heart infusion broth (HIB Difco) agar was used as solid complete medium Fluid complete medium was brain heart infusion broth (BH Difco) The basal media were those previously used (11)

Genetic Procedures

DNA preparation and transformation procedures followed the previously described technique (11) In the time course experiments the technique was slightly modified Phenotypically competent cells were obtained by inoculating the recipient strain in BH broth from an overnight culture on blood agar and following the absorbance until early logarithmic growth (11) The cells were harvested by centrifuging for 20 minutes at 4000 rpm and resuspended in saline to contain approximately 10^8 col forming units per ml 10 ml HIB with 0 005 M CaCl were inoculated with 1 ml of the suspension and incubated at 37 in a shaker for 20 minutes whereupon the appropriate inhibitor was added usually in a volume of 0 1 ml After 5 minutes 0 5 ml DNA dilution was added usually containing 50 µg/ml in NaCl citrate buffer Samples were removed at suitable intervals to tubes containing deoxyribonuclease (DNase) and $MgSO_4$ such that the final concentration of the enzyme was 50 µg/ml The DNase treated samples were assayed for transformants as previously described (11 14) The number of transformed units was calculated from counts on three plates made in parallel Suitable dilutions of the cultures were plated for the calculation of colony forming units Appropriate controls were included for revertants and for the detection of contamination Additional technical procedures or modifications have been described in the experimental section

Analysis of Covariation

In this paper the number of transformants obtained have been related to the time of DNA exposure This analysis of covariation followed the statistical principles previously used (10) When the graphs indicated that straight lines had been obtained the equations of the hypothetical lines which be t fitted the experimental results were determined by the method of least squares The coefficient of correlation (r) was calculated and discussed as to reality The standard error was calculated but since the actual ob ervation series usually consisted of less than 30 observations it had to be discussed according to the distribution of t The value $t = r/e_r$ was compared with a table showing the distribution of t If this preliminary analysis evinced no objection against the assumption that the experimental results represented the computed line the conformity of the experimental data with the calculated line was usually tested by the analysis of variance In order to do thi values to points in the regression line were calculated from the equation found (10)

RESULTS

Linear Accumulation of Singly Marked Transformants

Experiments were designed to study the appearance of *N meningitidis* transformants as a function of the duration of the contact of recipient cells and DNA Transformation of the mutants with Str r prototrophic DNA was first carried out at 37 Deoxyribonuclease was used to terminate the transformation It was observed that all types tested of singly marked transformants accumulate approximately linearly with duration of exposure to DNA as already shown for streptomycin restistance (5 20) When the curves are studied (Fig 1) it

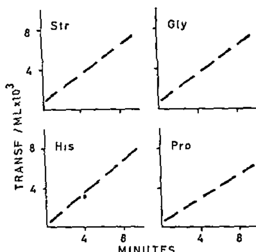


Fig 1

Linear accumulation of singly marked transformants in stirred mixtures of bacteria and DNA at 37 Calculated regression line for Str r is x = 1 44 y–1 01 for Gly x = 1 46 y–1 00 for His x = 1 28 y–0 26 for Pro x = 1 73 y–1 03

becomes likely that they extrapolate to a point before zero time i e to a time before the transforming DNA has actually been added From statistical analysis of covariation there appeared no objection against the assumption that the calculated straight lines represent the experimental values

Interference with the Linearity of the Uptake Curve

The kinetic curves of Fig 1 demonstrate the pattern observed when the recipient cells are in the early logarithmic phase of growth when DNA is added Manipulations which reduce the metabolic activity of the cells had a pronounced influence on the form of the curve When the metabolic activity was temporarily reduced by a cold shock before the addition of DNA a pronounced lag was obtained (Fig 2) during which a gradually increasing rate of appearance of transformants occurred

2 4 dinitrophenol is a potent inhibitor of oxidative phosphorylation with activity also on the metabolism of *N meningitidis* (13) Preincubation with 2 4 dinitrophenol had two effects on the transformation curve Fig 3 shows that a significant lag occurred before the appearance of the first transformants Furthermore the rate of appearance of transformants was reduced Further experiments showed that these effects were independent of any particular genetic trait among those tested but seemed to be characteristic of the *N meningitidis* transformation process in general when carried out under these conditions

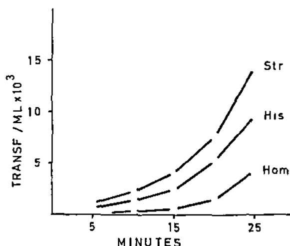


Fig 2

Interference with the linearity of the uptake curve by cold shock The recipient cells were cooled in ice water for 10 minutes and transferred to 37 DNA was added to the cells after temperature equilibration

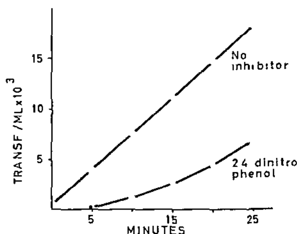


Fig 3

Interference with the kinetics of appearance of transformants to Str r by 1×10^{-3} M 2 4 dinitrophenol added 5 minutes before tran forming DNA

Establishment of a Lag Period Characterizing the Transformation Curve

To obtain further orientation concerning the more exact metabolic requirements for DNA uptake and transformation inhibitors of DNA replication and protein synthesis were tested

Hydroxyurea which is bacteriostatic in *E coli* seems to inhibit DNA synthesis in concentrations which do not affect RNA synthesis or protein metabolism (25 26) When competent cultures of *N meningitidis* in the early logarithmic growth phase are pretreated with hydroxyurea for 5 minutes before the addition of transforming DNA a significant

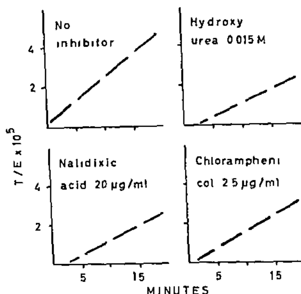


Fig 4

Reduction in the rate of appearance of transformants to Str r in the presence of hydroxyurea nalidixic acid or chloramphenicol The inhibitors were added 5 minutes before the transforming DNA

effect is observed in the kinetic curves (Fig 4) Furthermore it seems that all concentrations have the same approximate effect provided that they are not too high to permit a sufficient dilution of the chemical during the plating for essay of transformants This effect is a reduction in the rate of appearance of transformants to around one half of that found in the control system without hydroxyurea Calculations indicate that the experimental data obained in the presence of hydroxyurea may still be described by a linear regression line but when this line is extrapolated to zero transformants is is found that the point of intersection is at about 2 minutes Thus both the slope of the curve and the point of intersection has been changed This change in the kinetics seems to be common to all markers tested (Fig 5)

Nalidixic acid a potent inhibitor of DNA synthesis has a killing action on *E coli* But it has been shown that the inhibition can be reversed by transfer to a drug free medium provided that the transfer takes place within a fairly short period such as 60 minutes in *E coli* (6) From Fig 4 is seen that nalidixic acid added to competent N *meningitidis* cultures reduces the rate of appearance of transformants to approximately half of that in the control system Also in the presence of this inhibitor the point of intersection is moved to the right to approximately 2 minutes When nalidixic acid is added to the system it is particularly important to use concentrations which are sufficiently diluted out during the essay of transformants It is es

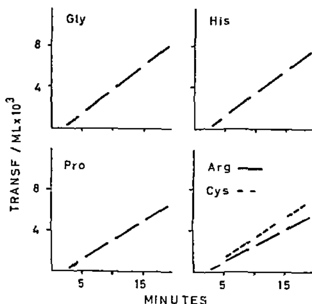


Fig 5

Lag period in the appearance of the first transformed marker in stirred mixtures of bacteria and transforming DNA in the presence of 0 015 M hydroxyurea Calculated regression line for Gly x = 2 14 y + 2 07 for His x = 2 56 y + 1 82 for Pro x = 2 98 y + 1 92 for Arg x = 3 16 y + 2 38 for Cys x = 2 54 y + 1 68

sential to use short times of exposure to the drug Even the concentration 20 µg/ml results in some killing particularly when the cells have been exposed to nalidixic acid for more than 10 minutes Therefore it becomes necessary to calculate the effiency of transformation T E in these experiments

Chloramphenicol was added in varying concentrations to competent cells of *N meningitidis* in the early logarithmic phase 5 minutes before the addition of DNA Fig 4 shows that also chloramphenicol has a significant effect on transformation with a reduction in the slope of the kinetics curve Even with chloramphenicol the point of intersection seems to be moved a little to the right resulting in a lag before the appearance of the first transformed marker

Since chloramphenicol and hydroxyurea seemed to have the same type of effect on the kinetics the combined effect was compared to that of each of the two drugs It seems that the combined effect is nearly the same as that of hydroxyurea alone while chloramphenicol alone is somewhat less effective (Fig 6)

Effect of the DNA Concentration on the Uptake Curve

The effect of DNA concentration on the kinetic curves obtained in the presence of inhibitors was examined From Fig 7 is seen that in the presence of hydroxyurea insignificant fluctuation occures in the

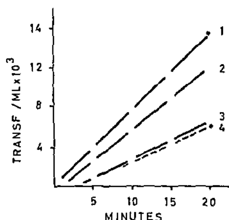


Fig 6

Combined effect of hydroxyurea and chloramphenicol on the rate of appearance of transformants to Str r Curve 1 Control system with no inhibitor Curve 2 2 μg chloramphenicol/ml Curve 3 0 015 M hydroxyurea Curve 4 2 μg chloramphenicol/ml plus 0 015 M hydroxyurea The inhibitors were added 5 minutes before the transforming DNA

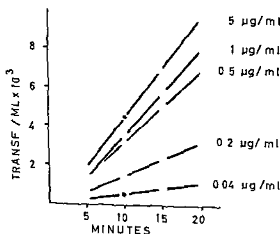


Fig 7

Formation of transformants to Str r in stirred mixtures of bacteria and transforming DNA at indicated concentrations The system was preincubated with 0 015 M hydroxyurea for 5 minutes before the addition of transforming DNA

lag period over a range of DNA concentration over 1000 An increasing rate of uptake was observed up to about 2 μg DNA per ml above which there is little further increase (Fig 8) Optimal concentrations of DNA are nearly the same as those obtained in *N meningitidis* without the addition of inhibitors (20) These results are not very different from those obtained in *H influenzae* (30)

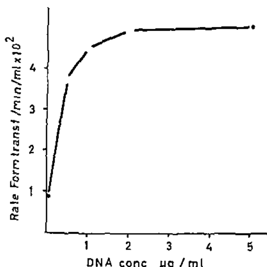


Fig 8

Relationship between rate of formation of transformants to Str r and concentration of transforming DNA in the presence of 0 015 M hydroxyurea

DISCUSSION

There are several arguments for a metabolically primed active trans port mechanism for DNA based on experiments with *Haemophilus* (2 30) and *B subtilis* (32) An energy source such as glucose is re quired and inhibitors of oxidative phosphorylation such as 2 4 dini trophenol or cyanide prevent irreversible uptake of DNA Also in *N meningitidis* an energy source is required for the uptake of trans forming DNA (20 K *Jyssum* Unpubl results)

The effects of 2 4 dinitrophenol on transformation in *N meningitidis* are similar to those observed in *H influenzae* (29 30) Stuy and Stern (30) assumed that the lag period and the decreased rate of DNA up take are both parameters for the uptake process and suggested that the chemical interferes with the flow of energy required for the DNA penetration step

But if energy production is necessary we need information regarding the particular functions for which the energy is required To obtain such information inhibitors of protein and DNA synthesis were tested During these experiments it has been assumed that the effects on the particular biosyntheses are the same in *N meningitidis* as those found in *E coli* (21 25 26 6)

There is a great deal of evidence indicating that DNA synthesis is not necessary for the uptake of DNA in *B subtilis* (22) or in pneu mococcus (7) The present experiments with hydroxyurea and nali dixic acid may be taken to indicate that also in *N meningitidis* no DNA synthesis is actually required

Preincubation of competent cells of *B subtilis* with high concentra

tions of chloramphenicol affects no aspect of the uptake kinetics (18) The effects of chloramphenicol reported above show that this drug cannot block the uptake of DNA during the *N meningitidis* transformation either implying that no protein synthesis is necessary

But even if nalidixic acid hydroxyurea and chloramphenicol do not stop transformation in *N meningitidis* they all bring about a significant reduction in the rate of appearance of transformants It may be assumed that this effect is primarily concerned with the metabolic situation which exists during the process of transformation

There are many indications that the competent state occurs when the cells have reached a resting state in most systems examined In *Haemophilus* as well as in *B licheniformis* competence seems to be induced by a down shift (17 29) Such procedures in meningococci only decrease the transformation frequencies (20) *Nester* (23) demonstrated that competent cells of *B subtilis* both before and after uptake of DNA are resistant to killing by penicillin for several hours implying that competent cells are neither multiplying nor synthesizing cell wall material Such differences have not been detected between competent and incompetent cells of *N meningitidis* (19)

Maximum competence in pneumococcus *H influenzae* and *B subtilis* is found in the latter part of the growth cycle and the beginning of the stationary phase (1 27 29) In contrast *N meningitidis* shows no sharp or high wave of competence The cells are transformed all through the growth cycle (5) but with maximum transformation efficiency in the early logarithmic phase (20)

In pneumococci there is a period of 1–2 hours between expression and onset of division of transformants (9) Newly transformed cells of *B subtilis* exhibit a lag in multiplication and expression of newly transformed markers of 3–4 hours (24 28) which cannot be attributed to lack of integration as donor DNA seems to become integrated within 30 to 45 minutes after it has been added (3 24 31) In meningococci there is virtually no lag between expression and onset of division (5 20)

Thus there are several indications which point to a high metabolic activity of the competent cells of meningococci The observed effects of hydroxyurea nalidixic acid and chloramphenicol could therefore mean that DNA replication protein synthesis as well as cell division actually take place during the process of transformation in this species although these functions are not required

The effect of chloramphenicol could indicate that enzymes which are active in the transformation are present at the time of DNA addition to competent cells but that they increase in quantity after the addition It could also mean that a new uptake process is induced in addition (16) Such a system does not seem to exist in *B subtilis* (18) On the other hand chloramphenicol does not seem to add to the inhibiting effect of hydroxyurea This may of course mean that the

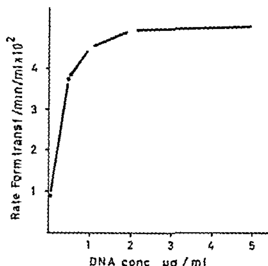


Fig 8

Relationship between rate of formation of transformants to Str r and concentration of transforming DNA in the presence of 0 015 M hydroxyurea

DISCUSSION

There are several arguments for a metabolically primed active transport mechanism for DNA based on experiments with *Haemophilus* (2 30) and *B subtilis* (32) An energy source such as glucose is required and inhibitors of oxidative phosphorylation such as 2 4 dinitrophenol or cyanide prevent irreversible uptake of DNA Also in *N meningitidis* an energy source is required for the uptake of transforming DNA (20 K *Jyssum* Unpubl results)

The effects of 2 4 dinitrophenol on transformation in *N meningitidis* are similar to those observed in *H influenzae* (29 30) Stuy and Stern (30) assumed that the lag period and the decreased rate of DNA uptake are both parameters for the uptake process and suggested that the chemical interferes with the flow of energy required for the DNA penetration step

But if energy production is necessary we need information regarding the particular functions for which the energy is required To obtain such information inhibitors of protein and DNA synthesis were tested During these experiments it has been assumed that the effects on the particular biosyntheses are the same in *N meningitidis* as those found in *E coli* (21 25 26 6)

There is a great deal of evidence indicating that DNA synthesis is not necessary for the uptake of DNA in *B subtilis* (22) or in pneumococcus (7) The present experiments with hydroxyurea and nalidixic acid may be taken to indicate that also in *N meningitidis* no DNA synthesis is actually required

Preincubation of competent cells of *B subtilis* with high concentra

interpreted to mean that in competent cells of this microbe DNA replication and cell division continue during the process of transformation although these functions are not actually required for the uptake of transforming DNA

REFERENCES

1 *Anagnostopoulos C & Spizizen J* Requirements for transformation in *Bacillus subtilis* J Bact *81* 741–746 1961

2 *Barnhart B J & Herriott R M* Penetration of deoxyribonucleic acid into *Hemophilus influenzae* Biochim biophys Acta (Amst) *76* 25–39 1963

3 *Bodmer W F & Ganesan A T* Biochemical and genetic studies of integration and recombination in *Bacillus subtilis* transformation Genetics *50* 717–738 1964

4 *Bovre K* Studies on transformation in *Moraxella* and organisms assumed to be related to *Moraxella* 1 A method for quantitative transformation in *Moraxella* and *Neisseria* with streptomycin resistance as the genetic marker Acta path microbiol scand *61* 457–473 1964

5 *Catlin B W* Transformation of *Neisseria meningitidis* by deoxyribonucleates from cells and from culture slime J Bact *79* 579 590 1960

6 *Deitz W H Cook T M & Goss W A* Mechanism of action of nalidixic acid on *Escherichia coli* III Conditions required for lethality J Bact *91* 768–773 1966

7 *Ephrussi Taylor H & Freed B A* Incorporation of thymidine and amino acids into deoxyribonucleic acid and acid insoluble cell structures in pneumococcal cultures synchronized for competence to transform J Bact. *87* 1211–1215 1964

8 *Green D M* Infectivity of DNA isolated from *Bacillus subtilis* bacteriophage SP82 J molec Biol *10* 438–451 1964

9 *Hotchkiss R D* Criteria for quantitative genetic transformation of bacteria In McElroy W D & Glass B (ed) The chemical basis of heredity p 321–335 Johns Hopkins Press Baltimore 1957

10 *Jyssum K* Indirect hemagglutinations by antigens from pneumococci Akademisk Trykningssentral Oslo 1955

11 *Jyssum K* *Polarity of chromosome replication in Neisseria meningitidis* J Bact *90* 1182–1187 1965

12 *Jyssum K* Genetic factors determining competence in transformation of *Neisseria meningitidis* 2 A reversible loss of competence Acta path microbiol scand *67* 493–502 1966

13 *Jyssum K & Borchgrevink B* The adaptive oxidation of l glutamic acid in meningococci Acta path microbiol scand *48* 361–366 1960

14 *Jyssum F & Jyssum S* Variation in density and transformation potential in deoxyribonucleic acid from *Neisseria meningitidis* J Bact *90* 1513 1519 1965

15 Kent L K & Hotchkiss R D Kinetic analysis of multiple linked recombinations in pneumococcal transformation J molec Biol *9* 308–322 1964

16 *Kohiyama M & Saito H* Stimulation of genetic transformation in *Bacillus subtilis* by biologically inactive DNA and polyphosphate Biochim biophys Acta (Amst) *41* 180–181 1960

17 *Leonard C G, Mattheis D K, Mattheis M J & Housewright R D* Transformation to prototrophy and polyglutamic acid synthesis in *Bacillus licheniformis* J Bact *88* 220–225 1964

18 *Levin J S & Strauss N* Lag period characterizing the entry of transforming deoxyribonucleic acid into *Bacillus subtilis* J Bact *89* 281–287 1965

19 *Lie S* On the effect of penicillin in the production of auxotrophic mutants of *Neisseria meningitidis* Acta path microbiol scand *63* 609–614 1965

20 *Lie S* Studies on the phenotypic expression of competence in *Neisseria meningitidis* Acta path microbiol scand *65* 119–129 1965

21 *Maaløe O & Hanawalt P C* Thymine deficiency and the normal DNA replication cycle I J molec Biol *3* 144–155 1961

latter agent also inhibits protein synthesis But if this is not the case one would expect that the common target is DNA synthesis Thus while hydroxyurea and nalidixic acid interfere with the DNA synthesis itself cloramphenicol may block the initiation of a new round of replication (21) There is also evidence that chloramphenicol has this effect in *N meningitidis* (*K Jyssum, J Bact 99* 757–763 1969) The difference between the chloramphenicol assay and the combined assay as well as the hydroxyurea experiment may then represent the completion of chromosome replication which had already been initiated before the addition of chloramphenicol

A fundamental difference between the transformation kinetics of *N meningitidis* without the addition of metabolic inhibitors and those exhibited by pneumococci (15) *B subtilis* (18) and *Haemophilus* (30) is the apparent lack of a lag period of entry of the first detectable marker when the transformation is terminated by deoxyribonuclease This pattern seems to be characteristic for *N meningitidis* (5 20) and other transformable *Neisseria* species as well as for *Moraxella* species (4)

It is easy to understand why a replication and a cell division during the time of observation may increase the slope of the transformation curve but it becomes necessary to explain why the lag period is obscured In the technique used nearly the same time lapsed between the addition of DNA and the final dilution and plating for all samples Since the cells are actively growing the chance of replication of a marker will be very high if it enters the cell early in the observation period and low if it enters late We may for instance assume that the hypothetical transformation curve $x = y + 1$ is obtained in the presence of inhibitor with x as the time before addition of deoxyribonuclease and y as the number of transformants obtained If one replication may take place of markers that have entered at the time $x = 2$ and the chance of replication is reduced to ½ for each successive time unit the following curve is obtained when the inhibitor is removed $x = \frac{3}{4} y + 1/$ Thus the slope as well as the point of intersection is changed

SUMMARY

The kinetics of appearance of transformants as a function of time of exposure to DNA has been studied in *Neisseria meningitidis* The addition of hydroxyurea or nalidixic acid as well as of chloramphenicol reduces the rate of formation of transformed cells A lag period appears before the first detected entry of each marker The length of this lag is approximately 2 minutes in the presence of hydroxyurea or nalidixic acid and seems to be independent of the genetic trait transformed The observed changes in kinetics have been discussed in relation to previous findings concerning the development of competence and the expression of transformed traits in *N meningitidis* The data have been

interpreted to mean that in competent cells of this microbe DNA replication and cell division continue during the process of transformation although these functions are not actually required for the uptake of transforming DNA

REFERENCES

1 *Anagnostopolos C & Spizizen J* Requirements for transformation in *Bacillus subtilis* J Bact *81* 741–746 1961

2 *Barnhart B J & Herriott R M* Penetration of deoxyribonucleic acid into *Hemophilus influenzae* Biochim biophys Acta (Amst) *76* 25–39 1963

3 *Bodmer W F & Ganesan A T* Biochemical and genetic studies of integration and recombination in *Bacillus subtilis* transformation Genetics *50* 717–738 1964

4 *Bövre K* Studies on transformation in *Moraxella* and organisms assumed to be related to *Moraxella* 1 A method for quantitative transformation in *Moraxella* and *Neisseria* with streptomycin resistance as the genetic marker Acta path microbiol scand *61* 457–473 1964

5 *Catlin B W* Transformation of *Neisseria meningitidis* by deoxyribonucleates from cells and from culture slime J Bact *79* 579 590 1960

6 *Deitz W H Cook T M & Goss W A* Mechanism of action of nalidixic acid on *Escherichia coli* III Conditions required for lethality J Bact *91* 768–773 1966

7 *Ephrussi Taylor H & Freed B A* Incorporation of thymidine and amino acids into deoxyribonucleic acid and acid insoluble cell structures in pneumococcal cultures synchronized for competence to transform J Bact *87* 1211–1215 1964

8 *Green D M* Infectivity of DNA isolated from *Bacillus subtilis* bacteriophage SP82 J molec Biol *10* 438–451 1964

9 *Hotchkiss R D* Criteria for quantitative genetic transformation of bacteria In McElroy W D & Glass B (ed) The chemical basis of heredity p 321–335 Johns Hopkins Press Baltimore 1957

10 *Jyssum K* Indirect hemagglutinations by antigens from pneumococci Akademisk Trykningssentral Oslo 1955

11 *Jyssum K* Polarity of chromosome replication in *Neisseria meningitidis* J Bact *90* 1180–1187 1965

12 *Jyssum K* Genetic factors determining competence in transformation of *Neisseria meningitidis* 2 A reversible loss of competence Acta path microbiol scand *67* 493–502 1966

13 *Jyssum K & Borchgrevink B* The adaptive oxidation of l glutamic acid in meningococci Acta path microbiol scand *48* 361–366 1960

14 *Jyssum K & Jyssum S* Variation in density and transformation potential in deoxyribonucleic acid from *Neisseria meningitidis* J Bact *90* 1513–1519 1965

15 *Kent J L & Hotchkiss R D* Kinetic analysis of multiple linked recombinations in pneumococcal transformation J molec Biol *9* 308 322 1964

16 *Kohiyama M & Saito H* Stimulation of genetic transformation in *Bacillus subtilis* by biologically inactive DNA and polyphosphate Biochim biophys Acta (Amst) *41* 180–181 1960

17 *Leonard C G Mattheis D K Mattheis M J & Housewright R D* Transformation to prototrophy and polyglutamic acid synthesis in *Bacillus licheniformis* J Bact *88* 220–225 1964

18 *Levine J S & Strauss N* Lag period characterizing the entry of transforming deoxyribonucleic acid into *Bacillus subtilis* J Bact *89* 281 287 1965

19 *Lie S* On the effect of penicillin in the production of auxotrophic mutants of *Neisseria meningitidis* Acta path microbiol scand *63* 603–614 1965

20 *Lie S* Studies on the phenotypic expression of competence in *Neisseria meningitidis* Acta path microbiol scand *64* 119–129 1965

21 *Maaløe O & Hanawalt P C* Thymine deficiency and the normal DNA replication cycle I J molec Biol *3* 144–155 1961

22 *McCarthy C & Nester E W* Macromolecular synthesis in newly transformed cells of *Bacillus subtilis* J Bact 94 131–140 1967
23 *Nester E W* Penicillin resistance of competent cells in deoxyribonucleic acid transformation of *Bacillus subtilis* J Bact 87 867–875 1964
24 *Nester E W & Stocker B A D* Biosynthetic latency in early stages of deoxyribonucleic acid transformation in *Bacillus subtilis* J Bact 86 785–796 1963
25 *Rosenkranz H S Garro A J Levy J A & Carr H S* Studies with hydroxyurea I The reversible inhibition of bacterial DNA synthesis and the effect of hydroxyurea on the bactericidal action of streptomycin Biochim biophys Acta (Amst) *114* 501–515 1966
26 *Rosenkranz H S Jacobs S J & Carr H S* Studies with hydroxyurea VIII The deoxyribonucleic acid of hydroxyurea treated cells Biochim biophys Acta (Amst) *161* 428–441 1968
27 *Schaeffer P* Transformation interspecifique chez des bacteries du genre *Hemophilus* Ann Inst Pasteur *91* 192–211 1956
28 *Stocker B A D* Transformation of *Bacillus subtilis* to motility and prototrophy Micromanipulative isolation of bacteria of transformed phenotype J Bact *86* 797–804 1963
29 *Stuy J H* Transformability of *Haemophilus influenzae* J gen Microbiol 29 537–549 1962
30 *Stuy J H & Stern D* The kinetics of DNA uptake by *Haemophilus influenzae* J gen Microbiol 35 391–400 1964
31 *Venema G Pritchard R H & Venema Schröder T* Fate of transforming deoxyribonucleic acid in *Bacillus subtilis* J Bact *89* 1250–1255 1965
32 *Young F E & Spizizen J* Physiological and genetic factors affecting transformation of *Bacillus subtilis* J Bact *81* 823–829 1961

Acta path. microbiol. scand 77 489–494 1969

The Department of Medical Microbiology University of Lund Lund Sweden

PRESENCE AND PERSISTENCE OF AUSTRALIAN ANTIGEN IN A SWEDISH HEPATITIS SERIES

By

E. NORDENFELT and L. KJELLÉN

Received 21 v 69

During a search for isoprecipitins in human sera *Blumberg et al* (1965) incidentally found a new serum protein The isoantigen has been called Australian antigen because it was first found in serum from an Australian aboriginal Later investigations (*Alter et al* 1966 *Blumberg et al* 1967) have shown that it is different from the lipoprotein isoantigen systems Australian antigen (Au) is rare (about 0 1 per cent) in normal American populations It often occurs transiently in patients with acute viral hepatitis (in 13 per cent with infectious hepatitis and in 34 per cent with post transfusion hepatitis) Au antigen also occurs in patients with some forms of leukaemia and Down s syndrome *Bayer et al* (1968) recently reported Au antigen in association with pleomorphic particles (about 200 Å) with a structure compatible with that of a virus

Prince (1968) has demonstrated an antigen termed SH that reacted in the immunodiffusion test with serum from multiply transfused patients The SH antigen was detected in blood during the incubation period before the onset of chemical or clinical signs of hepatitis Preliminary results suggested that the SH antigen is located on a virus like particle with an electron microscopic appearance similar to that of arbovirus and with a diameter of approximately 25 mμ SH antigen has been described as similar to Au antigen (*Prince* 1968)

Okochi & Murakami (1968) recently reported the occurrence of Au antigen in Japanese Like *Blumberg et al* and *Prince* they found Au antigen most often in patients with hepatitis (in 15 2 per cent with infectious hepatitis 12 9 per cent with post transfusion hepatitis) The frequency in blood donors in Tokyo was estimated at 1 per cent

The present study reports the frequency of Australian antigen in three selected hepatitis series in Sweden Blood specimens have been tested from cases treated at the clinic for Infectious diseases in Lund

We thank Dr *K Kaij* Dr *B Lindergårdh* for help with collecting sera and mrs *E. Miller* for skilful technical assistance.

The present work was supported by the Swedish Medical Research Council (project B 70-16x-2865-01)

22 *McCarthy C & Nester E W* Macromolecular synthesis in newly transformed cells of *Bacillus subtilis* J Bact 94 131–140 1967
23 *Nester E W* Penicillin resistance of competent cells in deoxyribonucleic acid transformation of *Bacillus subtilis* J Bact 87 867–875 1964
24 *Nester E W & Stocker B A D* Biosynthetic latency in early stages of deoxyribonucleic acid transformation in *Bacillus subtilis* J Bact 86 785–796 1963
25 *Rosenkranz H S Garro A J Levy J A & Carr H S* Studies with hydroxyurea I The reversible inhibition of bacterial DNA synthesis and the effect of hydroxyurea on the bactericidal action of streptomycin Biochim biophys Acta (Amst) 114 501–515 1966
26 *Rosenkranz H S Jacobs S J & Carr H S* Studies with hydroxyurea VIII The deoxyribonucleic acid of hydroxyurea treated cells Biochim biophys Acta (Amst) 161 428–441 1968
27 *Schaeffer P* Transformation interspecifique chez des bactéries du genre *Hemophilus* Ann Inst Pasteur 91 192–211 1956
28 *Stocker B A D* Transformation of *Bacillus subtilis* to motility and prototrophy Micromanipulative isolation of bacteria of transformed phenotype J Bact 86 797–804 1963
29 *Stuy J H* Transformability of *Haemophilus influenzae* J gen Microbiol 29 537–549 1962
30 *Stuy J H & Stern D* The kinetics of DNA uptake by *Haemophilus influenzae* J gen Microbiol 35 391–400 1964
31 *Venema G Pritchard R H & Venema Schroder T* Fate of transforming deoxyribonucleic acid in *Bacillus subtilis* J Bact 89 1250–1255 1965
32 *Young F E & Spizizen J* Physiological and genetic factors affecting transformation of *Bacillus subtilis* J Bact 81 823–829 1961

Acta path microbiol scand 77 489-494 1969

The Department of Medical Microbiology University of Lund Lund Sweden

PRESENCE AND PERSISTENCE OF AUSTRALIAN ANTIGEN IN A SWEDISH HEPATITIS SERIES

By

E NORDENFELT and L KJELLÉN

Received 21 v 69

During a search for isoprecipitins in human sera *Blumberg et al* (1965) incidentally found a new serum protein The isoantigen has been called Australian antigen because it was first found in serum from an Australian aboriginal Later investigations (*Alter et al* 1966 *Blumberg et al* 1967) have shown that it is different from the lipoprotein isoantigen systems Australian antigen (Au) is rare (about 0 1 per cent) in normal American populations It often occurs transiently in patients with acute viral hepatitis (in 13 per cent with infectious hepatitis and in 34 per cent with post transfusion hepatitis) Au antigen also occurs in patients with some forms of leukaemia and Down s syndrome *Bayer et al* (1968) recently reported Au antigen in association with pleomorphic particles (about 200 Å) with a structure compatible with that of a virus

Prince (1968) has demonstrated an antigen termed SH that reacted in the immunodiffusion test with serum from multiply transfused patients The SH antigen was detected in blood during the incubation period before the onset of chemical or clinical signs of hepatitis Preliminary results suggested that the SH antigen is located on a virus like particle with an electron microscopic appearance similar to that of arbovirus and with a diameter of approximately 25 mμ SH antigen has been described as similar to Au antigen (*Prince* 1968)

Okochi & Murakami (1968) recently reported the occurrence of Au antigen in Japanese Like *Blumberg et al* and *Prince* they found Au antigen most often in patients with hepatitis (in 15 2 per cent with infectious hepatitis 12 9 per cent with post transfusion hepatitis) The frequency in blood donors in Tokyo was estimated at 1 per cent

The present study reports the frequency of Australian antigen in three selected hepatitis series in Sweden Blood specimens have been tested from cases treated at the clinic for Infectious diseases in Lund

We thank Dr A *Kaij* Dr *B Lindergårdh* for help with collecting sera and mrs *E Miller* for skilful technical assistance

The present work was supported by the Swedish Medical Research Council (project B 70-16x-2865-01)

TABLE 1

The Appearance of Au Antigen before Signs of Clinical Hepatitis

Patient	Date	7/10	22/10	31/10	5/11	19/11	27/11	4/12	11/12
I B A	Au	—	—	—	+	+	+	+	+
	GPT	14	17	15	14	1[illegible]	40	*310*	[illegible]
	Date	4 12	11/12	18/12	27/12	3/1	14/1	[illegible]9/1	1[illegible]/2
B A	Au	—	—	—	+	+	+	+	+
	GPT	38	12	2[illegible]	19	20	14	*77*	[illegible]
	Date	18/12	27/12	3/1	14/1	[illegible]9/1	1[illegible]/2	3/3	
A S	Au	—	—	—	+	+	+	+	
	GPT	13	15	1[illegible]	9	10	9[illegible]	5[illegible]	

Abnormal GPT values are in italics

ber 1968 but when last tested two months later neither had clinical signs of hepatitis nor increased GPT been observed

It should be mentioned that in all the 14 patients who were receiving treatment with dialysis and in whom the reaction was or turned positive the reaction remained so throughout the investigation period Several have now exhibited Au antigen for at least 4 months

The 9 Au negative patients showed no signs of hepatitis during the investigation period One of them had hepatitis 2 months before the start of this investigation

The 4 cases of hepatitis among the staff who have been studied are included in the material from the clinic for infectious diseases Two of them have been Au positive

TABLE 2

Presence of Au Antigen in Patients Treated for Hepatitis on the Clinic for Infectious Diseases

		Cases	Au	Au
Clinical diagnose	Infectious hepatitis	8	2	6
	Serum hepatitis	13	8	5
		21	10	11

Au Antigen among Patients Treated at the Clinic for Infectious Diseases

Blood samples from most patients treated for hepatitis in the clinic for Infectious diseases have been tested for Au antigen since the summer 1968 Until now we have studied 40 sera from 21 patients The results are given in Table 2

The blood samples in this group were obtained in different stages of the disease In none of the ten Au positive cases were blood samples available from the early "incubation time From 7 of them however

the Medical clinic B (dialysis department) in Lund and the Juvenile Rehabilitation school at Raby (addicts)

MATERIAL AND METHODS

Clinical specimens Since October 1968 blood samples have been taken once a week from the 23 patients treated in the dialysis department

The blood specimens from patients treated for hepatitis at the unit for infectious diseases were taken in acute phase and/or in convalescens

Of 42 pupils at Råby in October 1968 28 were addicts In a clinical study of the occurrence of hepatitis among the pupils blood samples were taken at regular intervals

Reagents SH and Au antigen as well as positive antisera were generously placed at our disposal by Dr *A Prince* N Y and Dr *B Blumberg* Philadelphia

Method Double immunodiffusion in agarose according to *Ouchterlony* (1958) Agarose 0 9 per cent was used according to *Prince* (1968)

Glutamic pyruvic transaminase (GPT) The GPT activity in serum was determined fluormetrically in an autoanalyzer at the laboratory of Clinical Chemistry University Hospital Lund Values above 40 units are regarded as abnormal

Bilirubin Bilirubin in serum was determined in an autoanalyzer at the laboratory of Clinical Chemistry University Hospital Lund Values above 1 2 mg/100 ml are regarded as abnormal

Terminology Most of this work is done with reagents supplied by Dr Prince but we have for practical reasons preferred to refer to the antigen as Au antigen in accordance with the terminology of Blumberg et al In our hands reactions of identity were obtained when the reagents from the two laboratories were cross tested

RESULTS

Au Antigen among Patients Treated at the Dialysis Department

In the summer and fall of 1968 verified hepatitis occurred in patients or members of the staff Since then the patients and the personal have been regularly examined for increased GPT and presence of Au antigen The clinical symptoms of hepatitis were usually mild The diagnoses have been based on increased GPT

From Oct 1968–Jan 1969 105 sera from 23 patients receiving treatment with dialysis were examined for Au antigen Blood samples from 14 of these patients were found to contain Au antigen Judging from the GPT and other findings all except 1 of these 14 had or had shortly before had hepatitis

The Au positive cases can be divided into two groups one with Au antigen continously from the beginning and one where the test for Au turned positive during the investigation

The first group comprised 8 patients including 5 with clinical signs of hepatitis and increased GPT during the period of the investigation The other 3 had hepatitis with increased GPT 1–2 months before the investigation period

The second group consisted of 6 patients In three of these six did the Au antigen appear before signs of clinical hepatitis The results of the tests on these three patients are given in Table 1

In two of the six cases appearance of Au antigen was accompanied by an increase of the GPT The sixth case became Au positive in Decem

TABLE 1

The Appearance of Au Antigen before Signs of Clinical Hepatitis

Patient	Date	7/10	22/10	31/10	5/11	19/11	27/11	4/12	11/12
I B A	Au	—	—	—	+	+	+	+	+
	GPT	14	17	15	14	16	40	*310*	*351*
	Date	4/12	11/12	18/12	27/12	3/1	14/1	29/1	12/2
B A	Au	—	—	—	+	+	+	+	+
	GPT	38	12	22	19	20	14	77	55
	Date	18/12	27/12	3/1	14/1	29/1	12/2	3/3	
A S	Au	—	—	—	+	+	+	+	
	GPT	13	15	12	9	10	*95*	*56*	

Abnormal GPT values are in italics

ber 1968 but when last tested two months later neither had clinical signs of hepatitis nor increased GPT been observed

It should be mentioned that in all the 14 patients who were receiving treatment with dialysis and in whom the reaction was or turned positive the reaction remained so throughout the investigation period Several have now exhibited Au antigen for at least 4 months

The 9 Au negative patients showed no signs of hepatitis during the investigation period One of them had hepatitis 2 months before the start of this investigation

The 4 cases of hepatitis among the staff who have been studied are included in the material from the clinic for infectious diseases Two of them have been Au positive

TABLE 2

Presence of Au Antigen in Patients Treated for Hepatitis on the Clinic for Infectious Diseases

		Cases	Au	Au
Clinical diagnose	Infectious hepatitis	8	2	6
	Serum hepatitis	13	8	5
		21	10	11

Au Antigen among Patients Treated at the Clinic for Infectious Diseases

Blood samples from most patients treated for hepatitis in the clinic for Infectious diseases have been tested for Au antigen since the summer 1968 Until now we have studied 40 sera from 21 patients The results are given in Table 2

The blood samples in this group were obtained in different stages of the disease In none of the ten Au positive cases were blood samples available from the early incubation time From 7 of them however

the Medical clinic B (dialysis department) in Lund and the Juvenile Rehabilitation school at Råby (addicts)

MATERIAL AND METHODS

Clinical specimens Since October 1968 blood samples have been taken once a week from the 23 patients treated in the dialysis department

The blood specimens from patients treated for hepatitis at the unit for infectious diseases were taken in acute phase and/or in convalescens

Of 42 pupils at Råby in October 1968 28 were addicts In a clinical study of the occurrence of hepatitis among the pupils blood samples were taken at regular intervals

Reagents SH and Au antigen as well as positive antisera were generously placed at our disposal by Dr *A Prince* N Y and Dr *B Blumberg* Philadelphia

Method Double immunodiffusion in agarose according to *Ouchterlony* (1958) Agarose 0 9 per cent was used according to *Prince* (1968)

Glutamic pyruvic transaminase (GPT) The GPT activity in serum was determined fluormetrically in an autoanalyzer at the laboratory of Clinical Chemistry University Hospital Lund Values above 40 units are regarded as abnormal

Bilirubin Bilirubin in serum was determined in an autoanalyzer at the laboratory of Clinical Chemistry University Hospital Lund Values above 1 2 mg/100 ml are regarded as abnormal

Terminology Most of this work is done with reagents supplied by Dr Prince but we have for practical reasons preferred to refer to the antigen as Au antigen in accordance with the terminology of Blumberg et al In our hands reactions of identity were obtained when the reagents from the two laboratories were cross tested

RESULTS

Au Antigen among Patients Treated at the Dialysis Department

In the summer and fall of 1968 verified hepatitis occurred in patients or members of the staff Since then the patients and the personal have been regularly examined for increased GPT and presence of Au antigen The clinical symptoms of hepatitis were usually mild The diagnoses have been based on increased GPT

From Oct 1968–Jan 1969 105 sera from 23 patients receiving treatment with dialysis were examined for Au antigen Blood samples from 14 of these patients were found to contain Au antigen Judging from the GPT and other findings all except 1 of these 14 had or had shortly before had hepatitis

The Au positive cases can be divided into two groups one with Au antigen continously from the beginning and one where the test for Au turned positive during the investigation

The first group comprised 8 patients including 5 with clinical signs of hepatitis and increased GPT during the period of the investigation The other 3 had hepatitis with increased GPT 1–2 months before the investigation period

The second group consisted of 6 patients In three of these six did the Au antigen appear before signs of clinical hepatitis The results of the tests on these three patients are given in Table 1

In two of the six cases appearance of Au antigen was accompanied by an increase of the GPT The sixth case became Au positive in Decem

In two cases it has thus been possible to detect a transient occurrence of Au antigen In the other two the antigen was demonstrated in the first available blood sample in one (J V S) of them before the appearance of clinical and biochemical signs of hepatitis In all 4 cases the antigen disappeared while signs of hepatitis still persisted

DISCUSSION

Au antigen is very rare in normal populations in USA as well as in northern Europe (*Blumberg et al* 1968) Owing to shortage of antisera no attempts have been made to assess the frequency of Au antigen in Swedish populations

Our results confirm the earlier reports on Au antigen (*Blumberg et al* 1968 *Prince* 1968 *Okochi & Murakami* 1968) and its close association with serum hepatitis Of the 34 patients with serum hepatitis in our material 25 have been Au positive The association between Au antigen and serum hepatitis was thus high and practically 100 per cent in the patients receiving treatment with dialysis This is in good agreement with the finding by *London et al* (1968) In agreement with *Prince* (1968) and *Okochi & Murakami* (1968) the appearance of Au antigen has sometimes been demonstrated to precede the onset of clinical signs of hepatitis

In the hepatitis patients without complicating disorders the Au antigen was detected only transiently and disappeared earlier than the clinical signs of hepatitis This is in sharp contrast to the outcome obtained among patients receiving treatment with dialysis Once demonstrated the Au antigen did not disappear and all cases have remained Au positive during the 4–5 months investigation period The persistence of Au antigen may in some way be connected with uremia or the loss of normal renal function Of the 14 Au positive patients 6 have undergone bilateral nephrectomy the others were anuric All had been dialysed twice a week It is interesting to note that *Okochi & Murakami's* (1968) series included one uremia patient—still under observation—who had had the antigen for 5 months

Like *Blumberg et al* (1967) and *Okochi & Murakami* (1968) we have seen some Au positive cases among patients with the diagnosis of infectious hepatitis

The nature of Au antigen is still obscure *Bayer et al* (1968) as well as *Prince* (1968) have reported particles about 200 Å size with a structure compatible with that of a virus It might be mentioned that similar particles have been seen by us in an electron microscopical study The nature of these particles is receiving attention

SUMMARY

The occurrence of Au antigen among three different groups of patients has been studied A close association betw r Au antigen and hepatitis

repeated samples have been tested The antigen was demonstrable for only a short time despite persistance of clinical signs of hepatitis and increased GPT

TABLE 3

Raby School Pupils without Signs of Clinical Hepatitis Tested on One Occasion for Presence of Au Antigen and Antibodies

	No	Au	Au	Antibodies to Au antigen
Addicts	28	1	27	0
Alcoholics	12	0	12	0
Other	2	0	2	0
	42			

TABLE 4

Four Au Positive Addicts Tested Repeatedly During the Course of their Hepatitis for Presence of Au Antigen

Patient	Date	Au	Bilirubin mg/100 ml	GPT units†
KK	6/12	—	0 4	22
	27/12	+	3 3	1480
	10/1	—	2 4	1034
	25/2	—	0 8	30
KH	30/12	+	2 2	823
	9/1	+	8 5	1134
	24/1	—	3 5	517
	4/2	—	1 3	46
JAS	13/12	+	0 4	24
	2/1	+	0 8	550
	10/1	+	1 6	1144
	24/1	+	1 4	462
	5/2	—	0 9	87
TI	29/10	—	1 0	32
	5/2	+	6 5	682
	14/2	+	6 3	400
	6/3	—	1 2	48

Normal limit ≦ 1 2 mg/100 ml

† Normal limit ≦ 40 units

Au Antigen among Pupils at Raby School

Blood samples taken at one occasion from the pupils at the school were screened On that occasion none of the pupils showed overt clinical signs of hepatitis 42 pupils were tested (Table 3)

As seen from the table the reaction for Au was positive in 1 case

In the continued study 8 addicts were repeatedly examined in connection with the appearance of hepatitis Four of these were Au positive The results of the tests are given in Table 4

In two cases it has thus been possible to detect a transient occurrence of Au antigen In the other two the antigen was demonstrated in the first available blood sample in one (I A S) of them before the appearance of clinical and biochemical signs of hepatitis In all 4 cases the antigen disappeared while signs of hepatitis still persisted

DISCUSSION

Au antigen is very rare in normal populations in USA as well as in northern Europe (*Blumberg et al* 1968) Owing to shortage of antisera no attempts have been made to assess the frequency of Au antigen in Swedish populations

Our results confirm the earlier reports on Au antigen (*Blumberg et al* 1968 *Prince* 1968 *Okochi & Murakami* 1968) and its close association with serum hepatitis Of the 34 patients with serum hepatitis in our material 25 have been Au positive The association between Au antigen and serum hepatitis was thus high and practically 100 per cent in the patients receiving treatment with dialysis This is in good agreement with the finding by *London et al* (1968) In agreement with *Prince* (1968) and *Okochi & Murakami* (1968) the appearance of Au antigen has sometimes been demonstrated to precede the onset of clinical signs of hepatitis

In the hepatitis patients without complicating disorders the Au antigen was detected only transiently and disappeared earlier than the clinical signs of hepatitis This is in sharp contrast to the outcome obtained among patients receiving treatment with dialysis Once demonstrated the Au antigen did not disappear and all cases have remained Au positive during the 4–5 months investigation period The persistence of Au antigen may in some way be connected with uremia or the loss of normal renal function Of the 14 Au positive patients 6 have undergone bilateral nephrectomy the others were anuric All had been dialysed twice a week It is interesting to note that *Okochi & Murakami's* (1968) series included one uremia patient—still under observation—who had had the antigen for 5 months

Like *Blumberg et al* (1967) and *Okochi & Murakami* (1968) we have seen some Au positive cases among patients with the diagnosis of infectious hepatitis

The nature of Au antigen is still obscure *Bayer et al* (1968) as well as Prince (1968) have reported particles about 200 Å size with a structure compatible with that of a virus It might be mentioned that similar particles have been seen by us in an electron microscopical study The nature of these particles is receiving attention

SUMMARY

The occurrence of Au antigen among three different groups of patients has been studied A close association between Au antigen and hepatitis

is confirmed it was demonstrated in about 70 per cent of patients with serum hepatitis A persistence of Au antigen in patients receiving treatment with dialysis but not in others is found

REFERENCES

Alter H J & Blumberg B S Studies on a new human isoprecipitin system (Au stralia antigen) Blood 27 297–309 1966

Bayer H E Blumberg B S & Werner B Particles associated with Australia antigen in the sera of patients with Leukaemia Down s syndrome and Hepa titis Nature 218 1057–1059 1968

Blumberg B S Alter H J & Visnich S A new antigen in Leukaemia sera JAMA 191 541–546 1965

Blumberg B S Gerstley B J S Hungerford D A London W T & Sutnick A I A serum antigen (Australia antigen) in Down s syndrome Leukaemia and Hepatitis Ann Intern Med 66 924–931 1967

Blumberg B S Sutnick A I & London W T Hepatitis and Leukemia Their rela tion to Australia antigen Bull N Y Acad Med 44 1566–1584 1968

London W T Di Figlia M Sutnick A I Ziegenfuss J & Blumberg B S Austra lia antigen associated hepatitis epidemic in a hemodialysis unit The Au test Clin Res 16 567 1968

Okochi K & Murakami S Observations on Australia antigen in Japanese Vox Sang 15 374–385 1968

Ouchterlony O Diffusion—in—Gel Methods for Immunological Analysis Progr Allergy 5 1–78 1958

Prince A M Au antigen detected in the blood during the incubation period of serum hepatitis Proc N A S 60 814–821 1968

Prince A M Relation of Australian and SH antigens Lancet ii 462–463 1968

Acta path. microbiol scand 77 495 504 1969

The University of Bergen School of Medicine The Broegelmann Research Laboratory for Microbiology Bergen Norway

SEROLOGICAL PROPERTIES OF AQUEOUS ETHER EXTRACTED ENDOTOXIN FROM *NEISSERIA GONORRHOEAE*

By

J A MAELAND

Received 24 iv 69

Previous studies of various endotoxin preparations from a *N gonorrhoeae* strain using indirect haemagglutination techniques revealed two antigenic determinants (10) One of these determinants was of carbohydrate nature the other of protein nature They were designated determinant *a* and determinant *b* respectively Results obtained with the aqueous ether extract indicated that both determinants belong to the endotoxin complex (11) The aqueous ether endotoxin contained lipid sugar and protein the latter being the dominating component (12) Glucose galactose glucosamine and heptose were detected (12)

The present study of the aqueous ether endotoxin is concerned with the erythrocyte sensitizing activity the nature of the antibodies to the endotoxin and their reactivity in various tests

MATERIALS AND METHODS

N gonorrhoeae strain Strain 8551/64 was employed Cultivation and harvesting were performed as described previously (8)

Extraction method The bacteria were extracted with aqueous ether and the endotoxin was purified according to the procedures described previously (10 11)

Antisera Rabbit antisera to whole gonococci (anti Gc) were prepared as described earlier (8)

Antiserum to rabbit erythrocytes sensitized with alkali treated endotoxin (anti SRE) was prepared as follows The endotoxin was treated with 0 005 N NaOH at 37 C for 18 hrs neutralized and dialysed against 1/150 M phosphate buffered saline pH 7 2 Equal volumes of a solution containing 0 5 mg per ml of endotoxin and a one per cent suspension of erythrocytes from the animal to be immunized were mixed and kept at 37 C for 30 mins The erythrocytes were washed 5 times and prepared as a 20 per cent suspension in saline One ml was injected intravenously twice a week for 2 weeks and a last injection was given 4 weeks later

The animals were bled ? days after the last injection

Absorption of antisera Antibodies to both the determinants *a* and *b* were removed by absorption of undiluted antiserum with aqueous ether endotoxin Antisera containing antibodies to determinant *a* (anti Gc-*a* anti SRE *a*) but not to determinant *b* were prepared by absorption of the undiluted sera with gonococci treated with periodate (11) Antisera containing antibodies to determinant *b* (anti Gc *b* anti SRE *b*) but not to *a* were prepared by absorption with phenol water endotoxin (10 11)

That complete absorption of the antibodies had been secured was checked by

testing a 1 2 dilution of the absorbed era against appropriately sensitized ery throcytes (see below)

Absorption of anti Gc serum with erythrocytes sensitized with alkali treated endotoxin was performed by mixing one ml of packed sensitized erythrocytes with 1 6 ml serum diluted 1 32 The mixture was kept at 20 C for 30 mins followed by centrifugation at 1000 × *g* for 15 mins

Antiserum to rabbit serum A goat antiserum to whole rabbit serum was kindly provided by Dr *E Glück* Bergen The titre was 16 384 with sheep erythrocytes sensitized with sub agglutinating amounts of rabbit antiserum to sheep erythrocytes A 1 200 dilution of the goat antiserum was employed in the anti γ globulin test

Indirect haemagglutination and haemolysis inhibition of haemagglutination The antigen preparations used for sensitization of erythrocytes are described under Experiments and Results The techniques for the sensitization of erythrocytes and the performance of the tests have been described in earlier reports (8 10) In the indirect haemolysis test fresh guinea pig serum diluted 1 15 was employed as complement

Agar precipitation One per cent of agar (Special Noble) was used The antigen wells were filled with a suspension containing about 100 mg of wet gonococci per ml The suspension had been kept at 4 C for at least 3 days in order to obtain distinct lines in the precipitation test The serum wells were filled with undiluted antiserum The plates were incubated at 4 C for up to 10 days

Immunoelectrophoresis The equipment of LKB Produkter AB Stockholm Sweden was employed The saline suspension of gonococci was added to the antigen wells and a constant voltage of 250 V was applied for 90 mins at 20 C using a 0 06 M veronal buffer pH 8 6 Antiserum was then added and the reaction was observed for 2 days

Agglutination of gonococci Agglutination of untreated gonococcal cells and of gonococci heated at 100 C for 60 mins was performed in tubes Twofold serial dilutions of the antisera were prepared in phosphate buffered saline pH 7 2 in 0 2 ml volumes Equal volumes of a suspension of washed bacteria containing approximately 10^9 organisms per ml were added The tubes were incubated at 4 C for 18 hrs and centrifuged at 1000 × *g* for 60 secs the agglutination being recorded after resuspension of the sediment A control without antiserum was included

Complement fixation tests Twofold dilutions of the antiserum were prepared in 0 2 ml volumes Then 0 2 ml of appropriately diluted endotoxin and 0 2 ml containing 2 haemolytic units (100 per cent) of complement were added The mixtures were kept at 4 C for 18 hrs and then at 37 C for 10 mins Thereafter 0 4 ml of a one per cent suspension of erythrocytes sensitized with 2 haemolytic units (100 per cent) of rabbit antiserum to sheep erythrocytes was added After incubation at 37 C for 30 mins the degree of haemolysis was recorded and the results given as the reciprocal of the highest dilution with complete inhibition of haemolysis Antigen and serum controls were included Veronal buffer containing optimal amounts of Ca and Mg was used as diluent (4)

Gel filtration Undiluted antiserum 2 5 ml was subjected to gel filtration on columns of Sephadex G 200 according to the method of *Flodin & Killander* (3) The fractions were tested for activity in the indirect haemagglutination test

Reduction by mercaptoethanol Equal volumes of serum fractions from the Sephadex column and 0 2 M 2 Mercaptoethanol were mixed kept at 37 C for 30 mins and tested for activity without dialysis

Alkali and heat treatment of endotoxin Lyophilized endotoxin was dissolved in various concentrations of NaOH ranging from 0 0025 to 0 05 N The concentration of antigen was one mg per ml The solutions were kept at 37 C for 18 hrs neutralized with HCl dialysed against buffered saline and then adjusted to 0 5 mg per ml of antigen The preparations were used for sensitization of erythrocytes as described below

Heating was performed either in a boiling water bath for 60 mins or in the autoclave (120 C) for 60 mins

Digestion with pronase (B Grade Calbiochem) The digestion was carried out at 37 C for 4 hrs in phosphate buffered saline pH 7 2 with an enzyme to substrate ratio of 1 50 The enzyme was inactivated at 100 C for 5 mins

Oxidation with periodate The oxidation was carried out in the dark at 20 C for 20 hrs with 0 5 mg per ml of endotoxin in a solution of 0 01 M sodium metaperiodate

EXPERIMENTS AND RESULTS

Sensitization of Erythrocytes for Indirect Haemagglutination and Haemolysis

Attempts were made to sensitize normal sheep erythrocytes with solutions containing one mg per ml of untreated endotoxin Erythrocytes treated in this manner were not agglutinated by the anti Gc serum and haemolysis was not observed either when complement was added Apparently sensitization had not been achieved

Endotoxin heated at 100 C for 60 mins did not sensitize erythrocytes On the other hand sensitization was obtained with endotoxin heated at 120 C for 60 mins Solutions containing from 250 to 1000 µg per ml of the heated preparation sensitized erythrocytes for agglutination and haemolysis both with anti Gc *a* and with anti Gc *b* sera

Twofold serial dilutions of each sample of endotoxin treated with alkali (0 0025 to 0 05 N NaOH) were prepared and used for sensitization of equal volumes of a one per cent suspension of sheep erythrocytes Anti Gc *a* or anti Gc *b* sera 0 2 ml containing 8 agglutinating units were then added to 0 2 ml of a 0 5 per cent suspension of the sensitized erythrocytes In this way the minimal amount of antigen sensitizing erythrocytes for agglutination and for haemolysis with 8 units of the antisera could be determined

Anti Gc *a* and anti Gc *b* differed in their reactivity with sensitized erythrocytes depending on the concentration of NaOH used for treatment of the endotoxin (Fig 1) All samples of endotoxin treated with alkali sensitized erythrocyts for agglutination and haemolysis with anti Gc *b* The preparations treated with 0 01 or 0 02 N NaOH had the strongest ability to sensitize erythrocytes with determinant *b*

Anti Gc-*a* in contrast to anti Gc *b* did not agglutinate erythrocytes sensitized with endotoxin pretreated with 0 0025 to 0 01 N NaOH (Fig 1) Treatment with 0 01 N NaOH was necessary to induce haemolysis with anti Gc *a* Endotoxin pre treated with 0 02 to 0 05 N NaOH sensitized erythrocytes for agglutination and haeomlysis with anti Gc *a* the various samples showing almost identical sensitizing activity

The ability of the various alkali treated preparations to sensitize erythrocytes for haemolysis with anti Gc *a* or anti Gc *b* was markedly stronger than the ability to sensitize for agglutination especially with anti Gc *b* Results similar to those described were obtained when rabbit erythrocytes were used instead of sheep erythrocytes

Further experiments were performed to test whether antibodies to determinant *a* combined with erythrocytes sensitized with endotoxin which had been treated with 0 0025 to 0 01 N NaOH Erythrocytes sensitized with these preparations were incubated with 8 agglutinating units of the anti Gc *a* serum and thereafter washed three times The goat anti rabbit serum was then added No agglutination was observed indicating that rabbit antibodies had not combined with the sensitized

testing a 1 2 dilution of the absorbed sera again t appropriately sensitized ery throcytes (see below)

Absorption of anti Cc serum with erythrocytes sensitized with alkali treated endotoxin was performed by mixing one ml of packed sensitized erythrocytes with 1 6 ml serum diluted 1 32 The mixture was kept at 20 C for 30 mins followed by centrifugation at 1000 × *g* for 15 mins

Antiserum to rabbit serum A goat antiserum to whole rabbit serum was kindly provided by Dr *E Cluel* Bergen The titre was 16 384 with sheep erythrocytes sen sitized with sub agglutinating amounts of rabbit antiserum to sheep erythrocytes A 1 200 dilution of the goat antiserum was employed in the anti γ globulin test

Indirect haemagglutination and haemolysis inhibition of haemagglutination The antigen preparations used for sensitization of erythrocytes are described under Ex periments and Results The techniques for the sensitization of erythrocytes and the performance of the tests have been described in earlier reports (8 10) In the indirect haemolysis test fresh guinea pig serum diluted 1 15 was employed as complement

Agar precipitation One per cent of agar (Special Noble) was used. The antigen wells were filled with a suspension containing about 100 mg of wet gonococci per ml The suspension had been kept at 4 C for at least 3 days in order to obtain distinct lines in the precipitation test The serum wells were filled with undiluted antiserum The plates were incubated at 4 C for up to 10 days

Immunoelectrophoresis The equipment of LKB Produkter AB Stockholm Sweden was employed The saline suspension of gonococci was added to the antigen wells and a constant voltage of 250 V was applied for 90 mins at 20 C using a 0 06 M veronal buffer pH 8 6 Antiserum was then added and the reaction was observed for 2 days

Agglutination of gonococci Agglutination of untreated gonococcal cells and of gonococci heated at 100 C for 60 mins was performed in tubes Twofold serial dilutions of the antisera were prepared in phosphate buffered saline pH 7 2 in 0 2 ml volumes Equal volumes of a suspension of washed bacteria containing approximate ly 10^9 organisms per ml were added The tubes were incubated at 4 C for 18 hrs and centrifuged at 1000 × *g* for 60 secs the agglutination being recorded after resus pension of the sediment A control without antiserum was included

Complement fixation tests Twofold dilutions of the antiserum were prepared in 0 2 ml volumes Then 0 2 ml of appropriately diluted endotoxin and 0 2 ml con taining 2 haemolytic units (100 per cent) of complement were added The mixtures were kept at 4 C for 18 hrs and then at 37 C for 10 mins Thereafter 0 4 ml of a one per cent suspension of erythrocytes sensitized with 2 haemolytic units (100 per cent) of rabbit antiserum to sheep erythrocytes was added After incubation at 37 C for 30 mins the degree of haemolysis was recorded and the results given as the reciprocal of the highest dilution with complete inhibition of haemolysis Antigen and serum controls were included Veronal buffer containing optimal amounts of Ca and Mg^+ was used as diluent (4)

Gel filtration Undiluted antiserum 2 5 ml was subjected to gel filtration on columns of Sephadex G 200 according to the method of *Flodin & Killander* (3) The fractions were tested for activity in the indirect haemagglutination test

Reduction by mercaptoethanol Equal volumes of serum fractions from the Sephadex column and 0 2 M 2 Mercaptoethanol were mixed kept at 37 C for 30 mins and te ted for activity without dialysis

Alkali and heat treatment of endotoxin Lyophilized endotoxin was dissolved in various concentrations of NaOH ranging from 0 0025 to 0 05 N The concentration of antigen was one mg per ml The solutions were kept at 37 C for 18 hrs neutralized with HCl dialysed against buffered saline and then adjusted to 0 5 mg per ml of antigen The preparations were used for sensitization of erythrocytes a described below

Heating was performed either in a boiling water bath for 60 mins or in the auto clave (120 C) for 60 mins

Digestion with pronase (B Grade Calbiochem) The digestion was carried out at 37 C for 4 hrs in phosphate buffered saline pH 7 2 with an enzyme to substrate ratio of 1 50 The enzyme was inactivated at 100 C for 5 mins

Oxidation with periodate The oxidation was carried out in the dark at 20 C for 20 hrs with 0 5 mg per ml of endotoxin in a solution of 0 01 M sodium meta periodate

TABLE 2

Titres in Various Tests of Unabsorbed and Absorbed Antiserum to the Aqueous Ether Endotoxin

Test	Antigen	Anti SRE	Anti SRE *a*	Anti SRE *b*	X
Indirect haemaggl	Determinant *a*	512	512	<16	<16
Indirect haemolysis	Determinant *a*	4096	4096	<16	<16
Indirect haemaggl	Determinant *b*	1024	<16	1024	<16
Indirect haemolysis	Determinant *b*	16384	<16	8192	<16
Complement fixation	Endotoxin	256	128	128	<16
Bacterial aggl	Live gonococci	256	128	256	256
Bacterial aggl	Boiled gonococci	256	128	128	16

Anti SRE Antiserum to rabbit erythrocytes sensitized with the aqueous ether endotoxin
Anti SRE *a* Anti SRE after absorption of the antibodies to determinant *b*
Anti SRE *b* Anti SRE after absorption of the antibodies to determinant *a*
X Results obtained with the pre immune serum and with anti SRE absorbed with the endotoxin

shown in Table 2 Each antiserum agglutinated live and heat killed gonococci Absorption of anti SRE with endotoxin reduced its ability to agglutinate boiled gonococci to that of the pre immune serum showing that the endotoxin is an agglutinogen in heat treated bacteria On the other hand unabsorbed and absorbed anti SRE and the pre immune serum agglutinated live bacteria to the same titre Sera from 10 non immunized rabbits were examined for agglutination of gonococci When boiled gonococci were used the titres varied from 4 to 16 and when live bacteria were used from 128 to 256

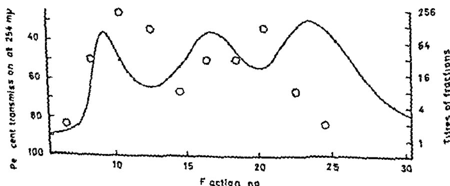


Fig 3

Titre in the indirect haemagglutination test of fraction obtained by gel filtration of rabbit anti Ce serum on Sephadex G 200 column Every two consecutive fractions 5 ml each were combined

—— Per cent transmission at 254 mμ
Titres of the antibodies to determinant *a*
○ Titres of the antibodies to determinant *b*

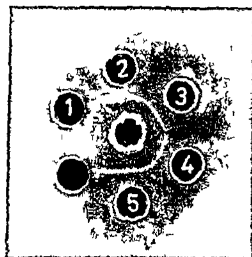


Fig 2
Precipitation pattern formed by gonococci and antisera
1 Anti SRE absorbed with the endotoxin 2 Anti SRE *b* 3 Anti SRE (unabsorbed) 4 Anti SRE *a* 5 Anti SRE *b*
Central well Gonococci

against running tap water and finally against buffered saline Usually 500 μg of the preparation was employed for sensitization of one ml of a one per cent suspension of erythrocytes Anti Gc *b* but not anti Gc *a* agglutinated erythrocytes sensitized in this manner

Either crude or purified endotoxin could be used to produce the sensitizing preparations

Activity in Various Tests of Anti SRE Anti SRE a and Anti SRE b, *and Nature of Antibodies to Determinants a and* b

In the ring precipitation test a solution containing endotoxin (on mg per ml) formed a precipitate against undiluted anti SRE up to a dilution of 1 32 and up to 1 16 against each of the sera anti SRE *a* and anti SRE-*b* In contrast the endotoxin formed no precipitation line with antisera in the gel diffusion tests (11) However each of the 3 antisera formed one precipitation line with a saline suspension of gonococci (Fig 2) the three precipitation lines fusing into one Anti SRE absorbed with the endotoxin gave no precipitation line with gonococci Using anti SRE or either of the specifically absorbed antisera in the antibody trough immunoelectrophoresis of the bacterial suspension showed a long precipitation arc which extended from the antigen well towards the anode

The results of indirect haemagglutination and haemolysis complement fixation and bacterial agglutination tests are compiled in Table 2 Anti SRE reacted with erythrocytes sensitized with determinant *a* and with determinant *b* The highest titres were obtained in the haemolysis test Anti SRE and each of the specifically absorbed anti sera fixed complement in the presence of the endotoxin It was found that at least 12 5 μg of antigen was required to obtain maximal titres in the complement fixation test when all 3 antisera gave the titres

TABLE 2

Titres in Various Tests of Unabsorbed and Absorbed Antiserum to the Aqueous Ether Endotoxin

Test	Antigen	Anti SRE	Anti SRE *a*	Anti SRE *b*	*
Indirect haemaggl	Determinant *a*	512	512	<16	<16
Indirect haemolysis	Determinant *a*	4096	4096	<16	<16
Indirect haemaggl	Determinant *b*	1024	<16	1024	<16
Indirect haemolysis	Determinant *b*	16384	<16	8192	<16
Complement fixation	Endotoxin	256	128	128	<16
Bacterial aggl	Live gonococci	256	128	256	256
Bacterial aggl	Boiled gonococci	256	128	128	16

Anti SRE Antiserum to rabbit erythrocytes sensitized with the aqueous ether endotoxin

Anti SRE *a* Anti SRE after absorption of the antibodies to determinant *b*

Anti SRE *b* Anti SRE after absorption of the antibodies to determinant *a*

* Results obtained with the pre immune serum and with anti SRE absorbed with the endotoxin

shown in Table 2 Each antiserum agglutinated live and heat killed gonococci Absorption of anti SRE with endotoxin reduced its ability to agglutinate boiled gonococci to that of the pre immune serum showing that the endotoxin is an agglutinogen in heat treated bacteria On the other hand unabsorbed and absorbed anti SRE and the pre immune serum agglutinated live bacteria to the same titre Sera from 10 non immunized rabbits were examined for agglutination of gonococci When boiled gonococci were used the titres varied from 4 to 16 and when live bacteria were used from 128 to 256

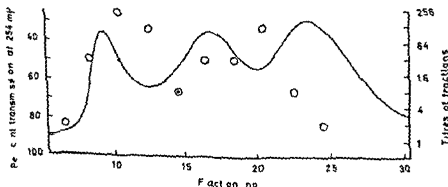


Fig 3

Titres in the indirect haemagglutination test of fractions obtained by gel filtration of rabbit anti SRE serum on Sephadex G 200 column Every two consecutive fractions 5 ml each were combined

—— Per cent transmission at 254 mμ

Titres of the antibodies to determinant *a*

○ Titres of the antibodies to determinant *b*

erythrocytes during incubation with anti Gc-*a*. Anti Gc serum was absorbed with erythrocytes sensitized with 0.005 N NaOH treated endotoxin. The titre of the antibodies to determinant *b* decreased from 4096 to less than 32, whereas the titre of the antibodies to determinant *a* was 1024 as in the case of the unabsorbed antiserum. Accordingly, antibodies to determinant *b* combined with the erythrocytes used for absorption, while the antibodies to determinant *a* did not.

In order to ascertain whether determinant *a* adhered to erythrocytes during incubation with the 0.005 N NaOH treated endotoxin, the alkali treated sample was absorbed repeatedly with sheep erythrocytes until the solution no longer sensitized erythrocytes for agglutination or haemolysis with anti Gc serum. However, the activity of determinants *a* and *b* measured by inhibition of haemagglutination was not significantly reduced by the absorptions. The results were thus inconclusive. On the other hand, this finding suggests that even after treatment with alkali only a number of the endotoxin particles have the capacity to adhere to erythrocytes. A rabbit was immunized with its own erythrocytes sensitized with the 0.005 N NaOH treated preparation. The antiserum obtained (anti SRE) contained antibodies to both *a* and *b* (see below). Accordingly, the erythrocytes had adsorbed both the determinants.

TABLE 1

Effect of Treatment with Alkali on the Activity of the Aqueous Ether Endotoxin Determined by Inhibition of Haemagglutination

Normality of NaOH	MID with erythrocytes sensitized with Determinant *a*	Determinant *b*
None	12.5	6.25
0.0025–0.02	12.5	6.25
0.03	12.5	50
0.04–0.05	12.5	>200

MID: Minimal inhibiting dose in µg with 8 agglutinating units of the antiserum.

The alkali treated preparations were tested for inhibition of haemagglutination using erythrocytes sensitized with determinant *a* or *b* according to the standard procedures described below. Treatment with alkali did not affect the inhibiting activity of determinant *a* (Table 1). The activity of determinant *b* was not changed by treatment with NaOH for up to 0.02 N, whereas treatment with 0.03 N NaOH reduced the activity and treatment with 0.04 and 0.05 N NaOH destroyed the activity of determinant *b* (Table 1). Anti Gc *b* apparently reacted with erythrocytes sensitized with samples of endotoxin treated with 0.04 and 0.05 N NaOH (see Fig. 1). This activity must be due to a determinant group different from *b*. The activity disappeared after digestion of the 0.04 and the 0.05 N NaOH treated preparations with pronase prior to sensitization of erythrocytes but not after periodate oxidation

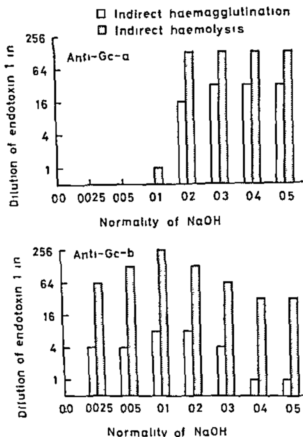


Fig 1

Erythrocyte sensitizing activity of aqueous ether endotoxin treated with various concentrations of NaOH Twofold dilutions of samples correspond ng to 1 mg per ml of endotoxin (ordinate) were used for sensitization Tested with 8 haemagglutinating units of anti Gc *a* (above) and anti Gc *b* (below) sera

As a standard procedure preparations sensitizing erythrocytes with determinant *a* were prepared as follows The endotoxin was treated with 0 04 N NaOH at 37 C for 18 hrs followed by neutralization with HCl and dialysis against phosphate buffered saline The protein component was then digested with pronase Four times the least amount of antigen which sensitized erythrocytes to maximal agglutination titres with antisera was employed for sensitization Thus from 125 to 250 μg of endotoxin was needed for sensitization of one ml of a one per cent suspension of erythrocytes Anti Gc *a* but not anti Gc *b* agglutinated the sensitized erythrocytes

Preparations sensitizing erythrocytes with determinant *b* were produced as follows The endotoxin was treated with 0 008 N NaOH at 37 C for 18 hrs followed by neutralization with HCl The preparation was then treated with periodate to destroy determinant *a* dialysed

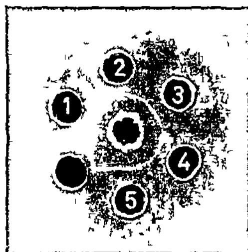


Fig 2
Precipitation pattern formed by gonococci and antisera
1 Anti SRE absorbed with the endotoxin 2 Anti SRE-*b* 3 Anti SRE (unabsorbed) 4 Anti SRE *a* 5 Anti SRE *b*
Central well Gonococci

against running tap water and finally against buffered saline Usually 500 μg of the preparation was employed for sensitization of one ml of a one per cent suspension of erythrocytes Anti Gc *b* but not anti Gc *a* agglutinated erythrocytes sensitized in this manner

Either crude or purified endotoxin could be used to produce the sensitizing preparations

Activity in Various Tests of Anti SRE Anti SRE a *and Anti SRE* b *and Nature of Antibodies to Determinants* a *and* b

In the ring precipitation test a solution containing endotoxin (one mg per ml) formed a precipitate against undiluted anti SRE up to a dilution of 1 32 and up to 1 16 against each of the sera anti SRE *a* and anti SRE *b* In contrast the endotoxin formed no precipitation line with antisera in the gel diffusion tests (11) However each of the 3 antisera formed one precipitation line with a saline suspension of gonococci (Fig 2) the three precipitation lines fusing into one Anti SRE absorbed with the endotoxin gave no precipitation line with gonococci Using anti SRE or either of the specifically absorbed antisera in the antibody trough immunoelectrophoresis of the bacterial suspension showed a long precipitation arc which extended from the antigen well towards the anode

The results of indirect haemagglutination and haemolysis complement fixation and bacterial agglutination tests are compiled in Table 2 Anti SRE reacted with erythrocytes sensitized with determinant *a* and with determinant *b* The highest titres were obtained in the haemolysis test Anti SRE and each of the specifically absorbed antisera fixed complement in the presence of the endotoxin It was found that at least 12 5 μg of antigen was required to obtain maximal titres in the complement fixation test when all 3 antisera gave the titres

TABLE 2

Titres in Various Tests of Unabsorbed and Absorbed Antiserum to the Aqueous Ether Endotoxin

Test	Antigen	Anti SRE	Anti SRE *a*	Anti SRE *b*	X
Indirect haemaggl	Determinant *a*	512	512	<16	<16
Indirect haemolysis	Determinant *a*	4096	4096	<16	<16
Indirect haemaggl	Determinant *b*	1024	<16	1024	<16
Indirect haemolysis	Determinant *b*	16384	<16	8192	<16
Complement fixation	Endotoxin	256	128	128	<16
Bacterial aggl	Live gonococci	256	128	256	256
Bacterial aggl	Boiled gonococci	256	128	128	16

Anti SRE Antiserum to rabbit erythrocytes sensitized with the aqueous ether endotoxin

Anti SRE *a* Anti SRE after absorption of the antibodies to determinant *b*

Anti SRE *b* Anti SRE after absorption of the antibodies to determinant *a*

X Results obtained with the pre immune serum and with anti SRE absorbed with the endotoxin

shown in Table 2 Each antiserum agglutinated live and heat killed gonococci Absorption of anti SRE with endotoxin reduced its ability to agglutinate boiled gonococci to that of the pre immune serum showing that the endotoxin is an agglutinogen in heat treated bacteria On the other hand unabsorbed and absorbed anti SRE and the pre immune serum agglutinated live bacteria to the same titre Sera from 10 non immunized rabbits were examined for agglutination of gonococci When boiled gonococci were used the titres varied from 4 to 16 and when live bacteria were used from 128 to 256

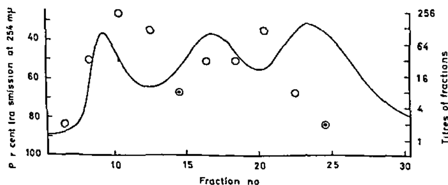


Fig 3

Titres in the indirect haemagglutination test of fractions obtained by gel filtration of rabbit anti Ce serum on Sephadex G 200 column Every two consecutive fractions 5 ml each were combined

——— Per cent transmission at 254 mμ
Titres of the antibodies to determinant *a*
○ Titres of the antibodies to determinant *b*

Anti SRE and anti Gc sera were subjected to gel filtration on Sephadex G 200 columns The haemagglutination titres of the fractions of the anti Gc serum were determined results are shown in Fig 3 The titres of the unfractionated antiserum were 512 and 1024 using the *a* and the *b* determinants respectively Antibodies to determinant *a* and to determinant *b* were found in the same fractions Treatment with mercaptoethanol completely destroyed the activity of fractions 6 to 10 but not the activity of fractions 11 to 24 Essentially the same results were obtained by Sephadex filtration of the anti SRE serum The results show that these antisera contained both γM and γG globulin antibodies to the determinants *a* and *b*

DISCUSSION

Endotoxin prepared from a *N gonorrhoeae* strain by extraction with aqueous ether required treatment with heat or alkali prior to sensitization of erythrocytes Phenol water lipopolysaccharides from gonococci (10) and other Gram negative cocci (6 7) as well as enterobacterial endotoxins (13) require similar treatment for sensitization While the effect on the endotoxin of such treatment was not investigated in the present study it has been correlated with the removal of some of the fatty acids or of O acetyl moieties (1 5)

Using different concentrations of NaOH for treatment of the endotoxin prior to sensitization it was shown that the availability of both the carbohydrate determinant (*a*) and the protein determinant (*b*) for their respective antibodies was affected

Anti Gc *b*, devoid of antibodies to determinant *a* reacted with erythrocytes sensitized with any of the alkali treated samples of endotoxin (Fig 1) However only samples treated with low concentrations of NaOH 0 03 N or less had the capacity to sensitize erythrocytes with determinant *b* since the antibody neutralizing ability of this determinant was destroyed by treatment with 0 04 or 0 05 N NaOH This means that the reaction of anti Gc *b* with erythrocytes sensitized with the 0 04 or 0 05 N NaOH treated preparations was due to a determinant group different from *b* probably one of protein nature It is possible that this determinant was made accessible by the alkali treatment

Endotoxin treated with 0 02 N or higher concentrations of NaOH sensitized erythrocytes for agglutination with antibodies to determinant *a* (Fig 1) Erythrocytes sensitized with samples of endotoxin treated with 0 01 N NaOH or less were not agglutinated Furthermore the results of the indirect haemolysis test the anti γ globulin test and the absorption experiment showed that the determinant *a* antibodies did not combine with these erythrocytes On the other hand the sensitized erythrocytes induced the formation in rabbits of antibodies to both *a* and *b* and therefore carried both the determinants Presumably some hindrance prevented the attachment of the *a* determinant antibodies

to erythrocytes sensitized with endotoxin which had been treated with low concentrations of NaOH The mechanism underlying this hindrance is not known

As indicated by results reported previously (10) erythrocytes sensitized with all ali extracted endotoxin acquire the specificity of determinant *b* Investigations have shown that anti Gc *b* but not anti Gc *a* combines with erythrocytes sensitized with the alkali endotoxin (*unpublished data*) However it has been shown by immunization experiments that during incubation with all ali extracted endotoxin both the determinants *a* and *b* adhered to the erythrocytes (9 10)

Due to the sensitizing properties of the aqueous ether endotoxin test systems for each of the determinants *a* and *b* could be developed This has been of great importance in the study of the endotoxin

Immunization of rabbits with erythrocytes sensitized with the aqueous ether endotoxin induced the formation of antibodies which could be detected by indirect haemagglutination and haemolysis precipitation complement fixation and bacterial agglutination tests This means that the antigenic determinants which attach to erythrocytes during incubation with the endotoxin have the capacity to participate in the various test systems Rabbit antibodies to the determinants *a* and *b* belong both to γM and to γG globulins analogous to rabbit antibodies to enterobacterial endotoxins (O antigens) (15)

Anti SRE and each of the specifically absorbed antisera (anti SRE *a* anti SRE *b*) formed one precipitation line against a suspension of bacterial cells These lines showed a reaction of identity in spite of the fact that determinant *a* and determinant *b* have different specificity However these determinants belong to one and the same molecular complex (11) a fact which might explain the precipitation pattern (14) On the other hand one or more determinants different from *a* and *b* may be involved in the precipitation reaction

The determinant groups present in the aqueous ether endotoxin function as agglutinogens in heated gonococci Using the slide agglutination test it has been observed that neither antiserum to the endotoxin (9) nor to heated gonococci (2) agglutinated live bacteria Therefore the tube agglutination technique was resorted to in this study It was however not possible to decide whether antibodies to the endotoxin have the capacity to agglutinate live gonococci since normal rabbit sera agglutinated live gonococci to comparatively high titres when this technique was used This agrees with the findings made by others (for references see 16) that sera from non immunized rabbits may contain antibodies reacting with gonococcal antigens

SUMMARY

Heat or alkali treatment of the aqueous ether endotoxin was necessary prior to sensitization of erythrocytes Both the carbohydrate and the

protein determinant of the endotoxin complex adsorbed to the erythrocytes However the different concentrations of sodium hydroxide used for treatment of the endotoxin prior to sensitization affected the availability of the carbohydrate and the protein determinant for their respective antibodies Techniques for sensitization of erythrocytes with each of these determinants are described

Antibodies to the endotoxin could be detected by indirect haemagglutination and haemolysis precipitation complement fixation and bacterial agglutination tests Rabbit antiserum to the endotoxin formed one precipitation line against whole gonococci Antibodies to the endotoxin belonged to γM and to γG globulins

REFERENCES

1 *Davies D A L Crumpton M J Macpherson I A & Hutchison A M* The adsorption of bacterial polysaccharides by erythrocytes Immunology *1* 157–171 1958

2 *Deacon W E Peacock W L Jr Freeman E M & Harris A* Identification of *Neisseria gonorrhoeae* by means of fluorescent antibodies Proc Soc exp Biol (N Y) *101* 322–325 1959

3 *Flodin P & Killander J* Fractionation of human serum proteins by gel filtration Biochim biophys Acta (Amst) *63* 403–410 1962

4 *Kabat E A & Mayer M M* Experimental immunochemistry Thomas Springfield Ill 1964 p 149

5 *Landy M Trapani R J & Clark W R* Studies on the O antigen of *Salmonella typhosa* III Activity of the isolated antigen in the hemagglutination procedure Amer J Hyg *62* 54–65 1955

6 *Mergenhagen S E Zipkin I & Varah E* Immunological and chemical studies on an oral *Veillonella* endotoxin J Immunol *88* 482–487 1962

7 *Mergenhagen S E Martin G R & Schiffmann E* Studies on an endotoxin of a group C *Neisseria meningitidis* J Immunol *90* 312–317 1963

8 *Mæland J A* Antibodies in human sera against antigens in gonococci demonstrated by a passive haemolysis test Acta path microbiol scand *67* 102–110 1966

9 *Mæland J A* Serological properties of antisera to *Neisseria gonorrhoeae* antigens Acta path microbiol scand *69* 145–155 1967

10 *Mæland J A* Antigenic properties of various preparations of *Neisseria gonorrhoeae* endotoxin Acta path microbiol scand *73* 413–422 1968

11 *Mæland J A* Antigenic determinants of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae* Acta path microbiol scand *76* 475–483 1969

12 *Mæland J A* Immunochemical characterization of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae* Acta path microbiol scand *76* 484–492 1969

13 *Neter E Westphal O Lüderitz O Gorzynski E A & Eichenberger E* Studies of enterobacterial lipopolysaccharides Effect of heat and chemicals on erythrocyte modifying antigenic toxic and pyrogenic properties J Immunol *76* 377–385 1956

14 *Ouchterlony Ö* Immunodiffusion and immunoelectrophoresis In Weir D M (Ed) Handbook of experimental immunology 1 ed Blackwell Scientific Publications Oxford and Edinburgh 1967 pp 655–706

15 *Pike R M* Antibody heterogeneity and serological reactions Bact Rev *31* 157–174 1967

16 *Reyn A* Serological studies on gonococci I Technique. Gono reaction of normal rabbits Serological relation between gonococci and pasteurellae Acta path microbiol scand 2[illegible] [illegible]1–70 1949

Acta path microbiol scand 77 505–517 1969

The University of Bergen School of Medicine The Broegelmann Research Laboratory for Microbiology Bergen Norway

SEROLOGICAL CROSS REACTIONS OF AQUEOUS ETHER EXTRACTED ENDOTOXIN FROM *NEISSERIA GONORRHOEAE* STRAINS

By

J A MAELAND

Received 24 iv 69

The results of chemical and serological analyses of endotoxin obtained by extraction of a *N gonorrhoeae* strain (strain 8551) with aqueous ether have been reported previously (18 19) The endotoxin was found to be composed of lipid carbohydrate and protein the latter being the major constituent (18) One antigenic determinant is attached to the carbohydrate component (determinant *a*) and another to the protein component (determinant *b*) (17) Techniques for sensitization of erythrocytes with each of these determinants have been described (19)

Aqueous ether endotoxin from three strains of gonococci has been analysed chemically and serologically particularly with respect to serological cross reactivity of the *a* and *b* determinants Other strains of gonococci and of Gram negative cocci belonging to other species have been tested for serological cross reactivity with the *a* and *b* determinants from the three strains of gonococci The results are presented in this paper

MATERIALS AND METHODS

Strains

N gonorrhoeae strain 8551 has been characterized and described in earlier reports (14 15 16 17 18 19) Strains V and VII were obtained from the Neisseria Department Statens Seruminstitut Copenhagen Denmark These strains are designated the *reference strains* Rabbit antisera to each of these strains (anti Gc 8551 anti Gc V and anti Gc VII) were prepared as described previously (14) Other strains of gonococci included were obtained from the National Collection of Type Cultures London from the Neisseria Department Statens Seruminstitut Copenhagen and from the Department of Microbiology the Gade Institute Bergen Four strains of *N meningitidis* 11 strains of apathogenic *Neisseria* species one strain of *Mima polymorpha* and 2 strains of *Herellea* species were obtained from the National Collection of Type Cultures London One strain of oral *Veillonella* was isolated in the Department of Microbiology the Gade Institute Bergen All strains were preserved and stored by lyophilization

Cultivation and Harvesting

The *Veillonella* strain was cultured anaerobically in an atmosphere of 93 per cent of hydrogen and 7 per cent of carbon dioxide on Bacto *Veillonella* Agar (Difco) All

Antiserum to each of the strains agglutinated erythrocytes sensitized with both the homologous and heterologous *a* and *b* determinants (Table 2) Compared with the titres obtained with the homologous *a* determinants the titres of the antisera were generally lower when tested with the heterologous *a* determinants The titres of the determinant *b* antibodies were the same irrespective of the strain from which the sensitizing antigen originated Pre immune sera diluted 1 16 gave no agglutination Accordingly the *a* determinants as well as the *b* determinants from the reference strains cross reacted serologically

The results of tests for inhibition of haemagglutination in which 16 agglutinating units of the antisera were used are compiled in Table 3 The endotoxin preparations gave inhibition in the homologous but not in the heterologous test systems for the *a* determinants Somewhat different results were obtained when 8 agglutinating units of the anti sera were employed Each endotoxin then inhibited the agglutination to some extent but not completely in heterologous systems for the *a* determinants with one exception Endotoxin VII did not at all inhibit the agglutination with anti Gc 8551 of erythrocytes sensitized with determinant *a* of endotoxin 8551

TABLE 3

Minimal Inhibiting Dose (MID in µg) of Aqueous Ether Endotoxin from N gonorrhoeae Strains 8551 X and VII Determined by Inhibition of Haemagglutination Erythrocytes Sensiti ed with Determinant a or Determinant b and Homologous Anti sera were Used as Test Systems

Preparation	MID in test system for					
	Determinant *a* of endotoxin			Determinant *b* of endotoxin		
	8551	X	VII	8551	X	VII
Endotoxin 8551	12 50	>200	>200	12 50	12 50	12 50
Endotoxin X	>200	6 25	>200	12 50	6 25	6 25
Endotoxin VII	>200	>200	12 50	12 50	6 25	6 25

Each endotoxin inhibited about equally effectively in homologous and heterologous test systems for the *b* determinants (Table 3) Apart from a difference in minimal inhibiting dose the results obtained were the same no matter whether 8 or 16 agglutinating units of the antisera were employed

The results of the inhibition tests indicate that the *b* determinants of the three endotoxins are identical with respect to specificity and that the *a* determinants are not identical

Previous to the absorption experiments the minimal amount of endotoxin neutralizing the *b* determinant antibodies in 0 2 ml of un diluted homologous antiserum was determined This amount of endo toxin was in excess of that required for neutralization of the *a* deter minant antibodies owing to the higher titres of the antibodies to deter

minant *b*. The homologous anti-Gc serum and each of the heterologous anti-Gc sera, 0.2 ml diluted 1:16, were absorbed with the estimated amount of endotoxin.

Absorption with homologous and heterologous endotoxin removed equally effectively the antibodies to determinant *b*. One absorption reduced the titres of the antisera from 2048 or more to less than 16. These findings agree with the results of the haemagglutination inhibition test and strongly indicate that the *b* determinants from the reference strains are identical.

TABLE 4

Titres in the Indirect Haemagglutination Test of Rabbit Antisera to N. gonorrhoeae Strains 8551, V and VII Absorbed with Endotoxins

Antiserum	Absorbed with	Erythrocytes sensitized with determinant *a* of endotoxin 8551	V	VII
Anti-Gc 8551	Endotoxin 8551	<16	<16	<16
	V	256	<16	<16
	VII	512	512	<16
Anti-Gc V	Endotoxin 8551	<16	1024	<16
	V	<16	<16	<16
	VII	256	1024	<16
Anti-Gc VII	Endotoxin 8551	<16	512	256
"	V	<16	<16	128
"	VII	<16	<16	<16

The titres of the unabsorbed antisera are shown in Table 2.

The effect of the absorptions on the titres of the *a* determinant antibodies is seen in Table 4. Absorption of homologous antiserum removed all the antibodies to determinant *a*. Absorption of heterologous antiserum removed the antibodies cross reacting with the *a* determinant used for absorption, but *a* determinant antibodies of other specificities were left unabsorbed, indicating multispecificity of the component hitherto called determinant *a*. The cross reactions between anti-Gc 8551 and determinant *a* of endotoxin VII and between anti-Gc VII and determinant *a* of endotoxin 8551 were eliminated by absorption with any of the endotoxins. Therefore these cross reactions must be due to an antigenic specificity common to the reference strains. Results similar to those shown in Table 4 were obtained by absorption with phenol-water lipopolysaccharide or lyophilized bacteria.

From Table 4 it is apparent that the various cross absorbed antisera and the sensitized erythrocytes which were agglutinated by these sera provided 9 test systems for haemagglutination inhibition experiments. The endotoxins were examined for inhibition in each of these test systems. The results presented further evidence of a multispecificity of the *a* determinants and showed most clearly the number of antigenic specificities (*a* factors) possessed by each of the reference

Antiserum to each of the strains agglutinated erythrocytes sensitized with both the homologous and heterologous *a* and *b* determinants (Table 2) Compared with the titres obtained with the homologous *a* determinants the titres of the antisera were generally lower when tested with the heterologous *a* determinants The titres of the determinant *b* antibodies were the same irrespective of the strain from which the sensitizing antigen originated Pre immune sera diluted 1 16 gave no agglutination Accordingly the *a* determinants as well as the *b* determinants from the reference strains cross reacted serologically

The results of tests for inhibition of haemagglutination in which 16 agglutinating units of the antisera were used are compiled in Table 3 The endotoxin preparations gave inhibition in the homologous but not in the heterologous test systems for the *a* determinants Somewhat different results were obtained when 8 agglutinating units of the anti sera were employed Each endotoxin then inhibited the agglutination to some extent but not completely in heterologous systems for the *a* determinants with one exception Endotoxin VII did not at all inhibit the agglutination with anti Gc 8551 of erythrocytes sensitized with determinant *a* of endotoxin 8551

TABLE 3

Minimal Inhibiting Dose (MID in μg) of Aqueous Ether Endotoxin from N gonorrhoeae Strains 8551 V and VII Determined by Inhibition of Haemagglutination Erythrocytes Sensitized with Determinant a *or Determinant* b *and Homologous Antisera were Used as Test Systems*

Preparation	MID in test system for					
	Determinant *a* of endotoxin			Determinant *b* of endotoxin		
	8551	V	VII	8551	V	VII
Endotoxin 8551	12 50	>200	>200	12 50	12 50	12 50
Endotoxin V	>200	6 25	>200	12 50	6 25	6 25
Endotoxin VII	>200	>200	12 50	12 50	6 25	6 25

Each endotoxin inhibited about equally effectively in homologous and heterologous test systems for the *b* determinants (Table 3) Apart from a difference in minimal inhibiting dose the results obtained were the same no matter whether 8 or 16 agglutinating units of the antisera were employed

The results of the inhibition tests indicate that the *b* determinants of the three endotoxins are identical with respect to specificity and that the *a* determinants are not identical

Previous to the absorption experiments the minimal amount of endotoxin neutralizing the *b* determinant antibodies in 0 2 ml of undiluted homologous antiserum was determined This amount of endotoxin was in excess of that required for neutralization of the *a* determinant antibodies owing to the higher titres of the antibodies to deter

minant *b* The homologous anti Gc serum and each of the heterologous anti Gc sera 0 2 ml diluted 1 16 were absorbed with the estimated amount of endotoxin

Absorption with homologous and heterologous endotoxin removed equally effectively the antibodies to determinant *b* One absorption reduced the titres of the antisera from 2048 or more to less than 16 These findings agree with the results of the haemagglutination inhibition test and strongly indicate that the *b* determinants from the reference strains are identical

TABLE 4

Titres in the Indirect Haemagglutination Test of Rabbit Antisera to N gonorrhoeae Strains 8551 V and VII Absorbed with Endotoxins

Antiserum	Absorbed with	Erythrocytes sensitized with determinant *a* of endotoxin 8551	V	VII
Anti Gc 8551	Endotoxin 8551	<16	<16	<16
"	V	*256*	<16	<16
	VII	*512*	*512*	<16
Anti Gc V	Endotoxin 8551	<16	*1024*	<16
	V	<16	<16	<16
	VII	*256*	*1024*	<16
Anti Gc VII	Endotoxin 8551	<16	*512*	*256*
	V	<16	<16	*128*
	VII	<16	<16	<16

The titres of the unabsorbed antisera are shown in Table 2

The effect of the absorptions on the titres of the *a* determinant antibodies is seen in Table 4 Absorption of homologous antiserum removed all the antibodies to determinant *a* Absorption of heterologous antiserum removed the antibodies cross reacting with the *a* determinant used for absorption but *a* determinant antibodies of other specificities were left unabsorbed indicating multispecificity of the component hitherto called determinant *a* The cross reactions between anti Gc 8551 and determinant *a* of endotoxin VII and between anti Gc VII and determinant *a* of endotoxin 8551 were eliminated by absorption with any of the endotoxins Therefore these cross reactions must be due to an antigenic specificity common to the reference strains Results similar to those shown in Table 4 were obtained by absorption with phenol water lipopolysaccharide or lyophilized bacteria

From Table 4 it is apparent that the various cross absorbed antisera and the sensitized erythrocytes which were agglutinated by these sera provided 9 test systems for haemagglutination inhibition experiments The endotoxins were examined for inhibition in each of these test systems The results presented further evidence of a multispecificity of the *a* determinants and showed most clearly the number of antigenic specificities (*a* factors) possessed by each of the reference

Antiserum to each of the strains agglutinated erythrocytes sensitized with both the homologous and heterologous *a* and *b* determinants (Table 2) Compared with the titres obtained with the homologous *a* determinants the titres of the antisera were generally lower when tested with the heterologous *a* determinants The titres of the determinant *b* antibodies were the same irrespective of the strain from which the sensitizing antigen originated Pre immune sera diluted 1 16 gave no agglutination Accordingly the *a* determinants as well as the *b* determinants from the reference strains cross reacted serologically

The results of tests for inhibition of haemagglutination in which 16 agglutinating units of the antisera were used are compiled in Table 3 The endotoxin preparations gave inhibition in the homologous but not in the heterologous test systems for the *a* determinants Somewhat different results were obtained when 8 agglutinating units of the antisera were employed Each endotoxin then inhibited the agglutination to some extent but not completely in heterologous systems for the *a* determinants with one exception Endotoxin VII did not at all inhibit the agglutination with anti Gc 8551 of erythrocytes sensitized with determinant *a* of endotoxin 8551

TABLE 3

Minimal Inhibiting Dose (MID in µg) of Aqueous Ether Endotoxin from N gonorrhoeae Strains 8551 X and VII Determined by Inhibition of Haemagglutination Erythrocytes Sensitized with Determinant a *or Determinant* b *and Homologous Antisera were Used as Test Systems*

Preparation	MID in test system for					
	Determinant a of endotoxin			Determinant b of endotoxin		
	8551	X	VII	8551	X	VII
Endotoxin 8551	12 50	>200	>200	12 50	12 50	12 50
Endotoxin X	>200	6 25	>200	12 50	6 25	6 25
Endotoxin VII	>200	>200	12 50	12 50	6 25	6 25

Each endotoxin inhibited about equally effectively in homologous and heterologous test systems for the *b* determinants (Table 3) Apart from a difference in minimal inhibiting dose the results obtained were the same no matter whether 8 or 16 agglutinating units of the antisera were employed

The results of the inhibition tests indicate that the *b* determinants of the three endotoxins are identical with respect to specificity and that the *a* determinants are not identical

Previous to the absorption experiments the minimal amount of endotoxin neutralizing the *b* determinant antibodies in 0 2 ml of undiluted homologous antiserum was determined This amount of endotoxin was in excess of that required for neutralization of the *a* determinant antibodies owing to the higher titres of the antibodies to deter

minant *b* The homologous anti Gc serum and each of the heterologous anti Gc sera 0 2 ml diluted 1 16 were absorbed with the estimated amount of endotoxin

Absorption with homologous and heterologous endotoxin removed equally effectively the antibodies to determinant *b* One absorption reduced the titres of the antisera from 2048 or more to less than 16 These findings agree with the results of the haemagglutination inhibition test and strongly indicate that the *b* determinants from the reference strains are identical

TABLE 4

Titres in the Indirect Haemagglutination Test of Rabbit Antisera to N gonorrhoeae *Strains 8551 V and VII Absorbed with Endotoxins*

Antiserum	Absorbed with	Erythrocytes sensitized with determinant *a* of endotoxin		
		8551	V	VII
Anti Gc 8551	Endotoxin 8551	<16	<16	<16
"	" V	256	<16	<16
	VII	512	512	<16
Anti Gc V	Endotoxin 8551	<16	1024	<16
	V	<16	<16	<16
	VII	256	1024	<16
Anti Gc VII	Endotoxin 8551	<16	512	256
	V	<16	<16	128
	VII	<16	<16	<16

The titres of the unabsorbed antisera are shown in Table 2

The effect of the absorptions on the titres of the *a* determinant antibodies is seen in Table 4 Absorption of homologous antiserum removed all the antibodies to determinant *a* Absorption of heterologous antiserum removed the antibodies cross reacting with the *a* determinant used for absorption but *a* determinant antibodies of other specificities were left unabsorbed indicating multispecificity of the component hitherto called determinant *a* The cross reactions between anti Gc 8551 and determinant *a* of endotoxin VII and between anti Gc VII and determinant *a* of endotoxin 8551 were eliminated by absorption with any of the endotoxins Therefore these cross reactions must be due to an antigenic specificity common to the reference strains Results similar to those shown in Table 4 were obtained by absorption with phenol water lipopolysaccharide or lyophilized bacteria

From Table 4 it is apparent that the various cross absorbed antisera and the sensitized erythrocytes which were agglutinated by these sera provided 9 test systems for haemagglutination inhibition experiments The endotoxins were examined for inhibition in each of these test systems The results presented further evidence of a multispecificity of the *a* determinants and showed most clearly the number of antigenic specificities (*a* factors) possessed by each of the reference

other strains were cultured on placental broth agar as described previously (14) The washed bacteria were stored frozen or lyophilized

Extraction Methods

Crude aqueous ether extracts were prepared from 27 strains of gonococci and from 4 strains of meningococci as described earlier (16) The aqueous ether endotoxin from the *N gonorrhoeae* strains 8551 V and VII (endotoxin 8551 endotoxin V and endotoxin VII) was purified by treatment of the crude extracts with DNase and repeated washings of the endotoxin with distilled water (17)

Phenol water lipopolysaccharides from strains 8551 V and VII were prepared as described earlier (16)

Serological Methods

Preparation of sensitized erythrocytes carrying determinant *a* or determinant *b* was performed as described earlier (19)

The indirect haemagglutination test and the test for inhibition of haemagglutination were performed according to previous reports (14 16) Unless otherwise stated 8 agglutinating units of the antisera were employed in the inhibition tests The minimal inhibiting dose (MID) is defined as the least amount of the preparation (in µg) which completely inhibits agglutination

Absorption of antiserum was performed either with endotoxin or with wet or lyophilized bacteria suspended in 1/150 M phosphate buffered saline pH 7 2 Lyophilized bacteria which did not form homogeneous suspensions were digested with trypsin (Trypure Novo) at 37 C for 2 hrs with an enzyme to sub trate ratio of 1 50 The enzyme was thereafter inactivated at 100 C for 5 mins The digestion invariably led to homogeneous bacterial suspensions Trypsin digestion and heating are without effect on determinants *a* and *b* (16) The absorptions were carried out at 4 C for 18 hrs followed by centrifugation at 5000 × *g* for 20 mins

Chemical Analyses

Protein was estimated by the Folin Ciocalteu method according to *Lowry et al* (12) with bovine serum albumin as standard and *nitrogen* by the micro Kjeldahl method (11) *Neutral sugar* was determined by the sulphuric acid orcinol method (28) with glucose galactose 1 1 as standard and *hexosamine* by the method of *Rondle & Morgan* (21) with glucosamine as standard *Heptose* was sought by the cysteine sulphuric acid reaction of *Dische* (8) *Total phosphorus* was determined by a modification of the method of *Fiske & Subbarow* (9 29) *Lipid* was determined spectrophotometrically as fatty acid ester groupings with tripalmitin as standard (22 25) *DNA* was estimated by the diphenylamine reaction of *Dische* (7) with DNA from calf thymus (Sigma Chemical Company) as standard

Paper chromatography for detection of sugar con tituents was performed as described earlier (18) The method included hydrolysis with 2 N H_2SO_4 at 100 C for 3 hrs neutralization with barium hydroxide centrifugation and lyophilization of the supernatant The sugars were then extracted with pyridine and subjected to circular paper chromatography in ethyl acetate pyridine water (40 11 6) The chromatograms were developed with the silver nitrate reagent of *Trevelyan et al* (26)

EXPERIMENTS AND RESULTS

Chemical Analyses of Endotoxin 8551 Endotoxin V and Endotoxin VII

The physicochemical properties of the three endotoxins were strikingly similar The preparations formed opalescent suspensions in water precipitated during treatment with DNase (pH 5 5) and were completely sedimented by centrifugation at 30 000 x *g* for 60 mins The lyophilized preparations were insoluble in water but dissolved readily in alkaline solutions

The quantitative chemical data a compiled in Table 1 Both the

Folin and the nitrogen values show that protein accounts for some 80 to 90 per cent of the preparations The amounts of neutral sugar and hexosamine were small less than 2 2 per cent of each Only endotoxin VII contained more than 3 per cent of fatty acids The data shown in Table 1 account for about 94 per cent of endotoxin 8551 and 88 and 92 per cent of endotoxin V and endotoxin VII respectively

TABLE 1

Chemical Composition of Aqueous Ether Endotoxin from N gonorrhoeae Strains 8551 V and VII (Per Cent of Dry Weight)

Preparation	N	Protein	P	Neutral sugar	Hexos amine	Lipid
Endotoxin 8551	14 10	88	0 35	1 80	1 12	3 00
Endotoxin V	12 60	82	0 30	2 16	1 00	2 60
Endotoxin VII	12 90	83	0 40	1 43	0 70	6 30

Glucose galactose and glucosamine were detected by paper chromatography of the hydrolysates The chromogens formed by the endotoxin preparations in the cysteine sulphuric acid reaction for heptoses gave nearly *identical absorption curves One absorption peak with maximum* at 390 mμ was due to the hexoses and a second peak with maximum at 505 mμ showed the presence of heptose (8)

The diphenylamine reaction for DNA was negative with endotoxin V and endotoxin VII whereas endotoxin 8551 contained one per cent of DNA

Serological Cross Reactions of Endotoxin 8551 Endotoxin V and Endotoxin VII

Both the *a* and *b* determinants of the endotoxin preparations were examined for cross reactivity in the indirect haemagglutination and the haemagglutination inhibition tests The antisera were used unabsorbed or absorbed with endotoxin

TABLE 2

Titres in the Indirect Haemagglutination Test of Rabbit Antisera to N gonorrhoeae Strains 8551 V and VII

Antiserum	Erythrocytes sensitized with Determinant *a* of endotoxin 8551	V	VII	Determinant *b* of endotoxin 8551	V	VII
Anti Gc 8551	1024	512	128	4096	4096	4096
Anti Gc V	512	2048	256	2048	2048	2048
Anti Gc VII	512	1024	1024	2048	2048	2048

Antiserum to each of the strains agglutinated erythrocytes sensitized with both the homologous and heterologous *a* and *b* determinants (Table 2). Compared with the titres obtained with the homologous *a* determinants, the titres of the antisera were generally lower when tested with the heterologous *a* determinants. The titres of the determinant *b* antibodies were the same irrespective of the strain from which the sensitizing antigen originated. Pre-immune sera diluted 1:16 gave no agglutination. Accordingly, the *a* determinants as well as the *b* determinants from the reference strains cross reacted serologically.

The results of tests for inhibition of haemagglutination in which 16 agglutinating units of the antisera were used are compiled in Table 3. The endotoxin preparations gave inhibition in the homologous but not in the heterologous test systems for the *a* determinants. Somewhat different results were obtained when 8 agglutinating units of the antisera were employed. Each endotoxin then inhibited the agglutination to some extent but not completely in heterologous systems for the *a* determinants, with one exception. Endotoxin VII did not at all inhibit the agglutination with anti-Gc 8551 of erythrocytes sensitized with determinant *a* of endotoxin 8551.

TABLE 3

Minimal Inhibiting Dose (MID in µg) of Aqueous Ether Endotoxin from N. gonorrhoeae Strains 8551, X and VII Determined by Inhibition of Haemagglutination Erythrocytes Sensitized with Determinant a *or Determinant* b *and Homologous Antisera were Used as Test Systems*

Preparation	MID in test system for Determinant *a* of endotoxin			Determinant *b* of endotoxin		
	8551	X	VII	8551	X	VII
Endotoxin 8551	12.50	>200	>200	12.50	12.50	12.50
Endotoxin X	>200	6.25	>200	12.50	6.25	6.25
Endotoxin VII	>200	>200	12.50	12.50	6.25	6.25

Each endotoxin inhibited about equally effectively in homologous and heterologous test systems for the *b* determinants (Table 3). Apart from a difference in minimal inhibiting dose, the results obtained were the same no matter whether 8 or 16 agglutinating units of the antisera were employed.

The results of the inhibition tests indicate that the *b* determinants of the three endotoxins are identical with respect to specificity and that the *a* determinants are not identical.

Previous to the absorption experiments, the minimal amount of endotoxin neutralizing the *b* determinant antibodies in 0.2 ml of undiluted homologous antiserum was determined. This amount of endotoxin was in excess of that required for neutralization of the *a* determinant antibodies owing to the higher titres of the antibodies to deter-

minant *b* The homologous anti Gc serum and each of the heterologous anti Gc sera 0 2 ml diluted 1 16 were absorbed with the estimated amount of endotoxin

Absorption with homologous and heterologous endotoxin removed equally effectively the antibodies to determinant *b* One absorption reduced the titres of the antisera from 2048 or more to less than 16 These findings agree with the results of the haemagglutination inhibition test and strongly indicate that the *b* determinants from the reference strains are identical

TABLE 4

Titres in the Indirect Haemagglutination Test of Rabbit Antisera to N gonorrhoeae Strains 8551 X and XII Absorbed with Endotoxins

Antiserum	Absorbed with	Erythrocytes sensitized with determinant *a* of endotoxin		
		8551	X	XII
Anti Gc 8551	Endotoxin 8551	<16	<16	<16
	X	256	<16	<16
	XII	*512*	*512*	<16
Anti Gc X	Endotoxin 8551	<16	*1024*	<16
	X	<16	<16	<16
	XII	*256*	*1024*	<16
Anti Gc XII	Endotoxin 8551	<16	*512*	*256*
	X	<16	<16	*128*
"	XII	<16	<16	<16

The titres of the unabsorbed antisera are shown in Table 2

The effect of the absorptions on the titres of the *a* determinant antibodies is seen in Table 4 Absorption of homologous antiserum removed all the antibodies to determinant *a* Absorption of heterologous antiserum removed the antibodies cross reacting with the *a* determinant used for absorption but *a* determinant antibodies of other specificities were left unabsorbed indicating multispecificity of the component hitherto called determinant *a* The cross reactions between anti Gc 8551 and determinant *a* of endotoxin XII and between anti Gc XII and determinant *a* of endotoxin 8551 were eliminated by absorption with any of the endotoxins Therefore these cross reactions must be due to an antigenic specificity common to the reference strains Results similar to those shown in Table 4 were obtained by absorption with phenol water lipopolysaccharide or lyophilized bacteria

From Table 4 it is apparent that the various cross absorbed antisera and the sensitized erythrocytes which were agglutinated by these sera provided 9 test systems for haemagglutination inhibition experiments The endotoxins were examined for inhibition in each of these test systems The results presented further evidence of a multispecificity of the *a* determinants and showed most clearly the number of antigenic specificities (*a* factors) possessed by each of the reference

strains Table 5 shows the results obtained in the 6 test systems necessary for the demonstration of these factors The agglutination with unabsorbed anti Gc VII of erythrocytes sensitized with determinant a of endotoxin 8551 was inhibited by each of the endotoxins Therefore this inhibition was due to a factor shared by the endotoxins This factor is designated a_1 Either one or two of the endotoxin preparations gave inhibition in the 5 remaining test systems reflecting a factors possessed by only one of the preparations or factors shared by two of the preparations Only strain V possessed the factor called a Another factor called a_3 was shared by strain V and strain VII Only strain VII carried the factor called a_4 and factor a_5 occurred only in strain 8551 Strains 8551 and V had in common the factor called a_6 Since the b determinants of the reference strains have identical specificity the tentative antigenic formulas are as follows Endotoxin 8551 $ba_{1,5,6}$ Endotoxin V $ba_{1,\ 3,6}$ Endotoxin VII $ba_{1,3,4}$ Endotoxin 8551 and endotoxin VII have in common only factor a_1 whereas endotoxin V has two a factors in common with each of the other preparations Each endotoxin contained one a factor which was not present in the other preparations

It was found that most anti Gc 8551 sera contained antibodies of very low titres to factor a_1 Only one of 5 different sera tested for agglutination of erythrocytes sensitized with determinant a of endotoxin VII (a_1) gave a titre of 128 whereas the titres of the remaining sera varied from 16 to 32 Anti Gc V serum contained no antibodies to factor a_3 Nevertheless both the absorption experiments and the inhibition tests have shown that endotoxin V possessed factor a_3

Distribution of the a *Factors and of Determinant* b *among Gonococci and Gram Negative Cocci of other Species*

Samples of 200 and 100 µg of crude aqueous ether endotoxin prepared from 24 strains of gonococci and 4 strains of meningococci were

TABLE 5

Minimal Inhibiting Dose (MID in µg) of Aqueous Ether Endotoxin from N gonorrhoeae Strains 8551 V and VII Determined by Inhibition of Haemagglutination with Unabsorbed and Absorbed Antisera.

Test system made up of: Anti-Gc	Determinant a of	MID of endotoxin: 8551	V	VII	Factor designation
VII unabs.	End -8551	6.25	6.25	12.50	a_1
V abs. end. 8551	End. V	>[illegible]	3.12	[illegible]	a
VII abs. end. 8551	End. V	[illegible]	1.56		a_3
VII abs. end. V	End. VII	[illegible]	>[illegible]		a
-8551 abs. end. V	End.	[illegible]	>200		a_5
-8551 abs. end. VII	End. V	[illegible]	[illegible]		a

Anti-Gc Antiserum [illegible]

TABLE 6

Distribution of Determinant b and of the Factors of Determinant a in N gonorrhoeae and N meningitidis Strains Determined by Inhibition of Haemagglutination with Aqueous Ether Extracts

Strains	Factors of determinant a						Determinant b
	a_1	a_2	a_3	a_4	a_5	a_6	
N gonorrhoeae							
SS 117624 SS 117573 SS 1 0469	+	—	—	—	—	+	+
CI 8116 SS VII	+	—	+	+	—	—	+
CI 6976	+	—	—	+	—	+	+
CI 7471 CI 8551 SS 120709 SS 126779	+	—	—	—	+	+	+
CI 7174 CI 8172 CI 9149 SS V SS 124392 SS 126492	+	+	+	—	—	+	+
CI 6115 GI 6526 SS III	+	+	—	—	+	+	+
SS 126f76	+	—	+	+	—	+	+
SS 117579	+	—	—	+	+	+	+
NCTC 8?0	+	+	+	+	—	+	+
SS 1 6589 SS 126773 SS 126890 SS 126879	+	+	+	—	+	+	+
NCTC 71 9	+	+	—	+	+	+	+
N meningitidis							
group A NCTC 10025	—	—	—	—	—	+	+
group B NCTC 10026	+	—	—	—	—	+	+
group C, NCTC 8554	+	—	—	—	—	—	+
group D NCTC 6457	—	—	—	—	—	—	+

SS The Neisseria Department Statens Seruminstitut Copenhagen
GI The Department of Microbiology the Gade Institute Bergen
NCTC The National Collection of Type Cultures London
+ and — indicate presence or absence of the *a* factors and determinant *b*

strains Table 5 shows the results obtained in the 6 test systems necessary for the demonstration of these factors The agglutination with unabsorbed anti Gc VII of erythrocytes sensitized with determinant a of endotoxin 8551 was inhibited by each of the endotoxins Therefore this inhibition was due to a factor shared by the endotoxins This factor is designated a_1 Either one or two of the endotoxin preparations gave inhibition in the 5 remaining test systems reflecting a factors possessed by only one of the preparations or factors shared by two of the preparations Only strain V possessed the factor called a Another factor called a_3 was shared by strain V and strain VII Only strain VII carried the factor called a_4 and factor a_5 occurred only in strain 8551 Strains 8551 and V had in common the factor called a_6 Since the b determinants of the reference strains have identical specificity the tentative antigenic formulas are as follows Endotoxin 8551 $ba_{1\ 5,6}$ Endotoxin V $ba_{1\ \ 3\ 6}$ Endotoxin VII $ba_{1,3,4}$ Endotoxin 8551 and endotoxin VII have in common only factor a_1 whereas endotoxin V has two a factors in common with each of the other preparations Each endotoxin contained one a factor which was not present in the other preparations

It was found that most anti Gc 8551 sera contained antibodies of very low titres to factor a_1 Only one of 5 different sera tested for agglutination of erythrocytes sensitized with determinant a of endotoxin VII (a_1) gave a titre of 128 whereas the titres of the remaining sera varied from 16 to 32 Anti Gc V serum contained no antibodies to factor a_3 Nevertheless both the absorption experiments and the inhibition tests have shown that endotoxin V possessed factor a_3

Distribution of the a Factors and of Determinant b *among Gonococci and Gram Negative Cocci of other Species*

Samples of 200 and 100 µg of crude aqueous ether endotoxin prepared from 24 strains of gonococci and 4 strains of meningococci were

TABLE 5

Minimal Inhibiting Dose (MID in µg) of Aqueous Ether Endotoxin from N gonorrhoeae Strains 8551 V and VII Determined by Inhibition of Haemagglutination with Unabsorbed and Absorbed Antisera

Test system made up of: Anti Gc	Determinant a of	MID of endotoxin: 8551	V	VII	Factor designation
VII unabs	End 8551	6 25	6 2	12 50	a_1
V abs end 8551	End V	>200	3 12	>200	a
VII abs end 8551	End V	>200	1 56	12 50	a_3
VII abs end. V	End. VII	>200	>200	12 50	a_4
8551 abs end. V	End 8551	3 12	>200	>200	a
8551 abs end VII	End V	6 25	12 50	>200	a_6

Anti Gc Antiserum to whole gonococci

TABLE 6

Distribution of Determinant b and of the Factors of Determinant a in N gonorrhoeae and N meningitidis Strains Determined by Inhibition of Haemagglutination with Aqueous Ether Extracts

Strains	a_1	a	a_3	a_4	a_5	a_6	Determinant b
	Factors of determinant a						
N gonorrhoeae							
SS 119694 SS 117573 SS 120469	+	−	−	−	−	+	+
GI 8116 SS VII	+	−	+	+	−	−	+
CI 6976	+	−	−	+	−	+	+
GI 7471 GI 8551 SS 120709 SS 126772	+	−	−	−	+	+	+
CI 7174 CI 8172 CI 9142 SS V SS 124392 SS 126492	+	+	+	−	−	+	+
GI 6115 CI 6576 SS III	+	+	−	−	+	+	+
SS 120676	+	−	+	+	−	+	+
SS 117579	+	−	−	+	+	+	+
NCTC 6890	+	+	+	+	−	+	+
SS 126589 SS 126773 SS 126890 SS 126899	+	+	+	−	+	+	+
NCTC 7129	+	+	−	+	+	+	+
N meningitidis							
group A NCTC 10025	−	−	−	−	−	+	+
group B NCTC 10026	+	−	−	−	−	+	+
group C NCTC 8554	+	−	−	−	−	−	+
group D NCTC 6457	−	−	−	−	−	−	+

SS The Neisseria Departme t Statens Seruminstitut Copenhagen
CI The Department of M c obiolog the Gade Institute Bergen
NCTC The National Collection of Type Cultures London
+ and − indi ate pre ence or absence of the a factors and determinant b

strains Table 5 shows the results obtained in the 6 test systems necessary for the demonstration of these factors The agglutination with unabsorbed anti Gc VII of erythrocytes sensitized with determinant a of endotoxin 8551 was inhibited by each of the endotoxins Therefore this inhibition was due to a factor shared by the endotoxins This factor is designated a_1 Either one or two of the endotoxin preparations gave inhibition in the 5 remaining test systems reflecting a factors possessed by only one of the preparations or factors shared by two of the preparations Only strain V possessed the factor called a Another factor called a_3 was shared by strain V and strain VII Only strain VII carried the factor called a_4 and factor a_5 occurred only in strain 8551 Strains 8551 and V had in common the factor called a_6 Since the b determinants of the reference strains have identical specificity the tentative antigenic formulas are as follows Endotoxin 8551 $ba_{1,5,6}$ Endotoxin V $ba_{1,\ 3,6}$ Endotoxin VII $ba_{1,3,4}$ Endotoxin 8551 and endotoxin VII have in common only factor a_1 whereas endotoxin V has two a factors in common with each of the other preparations Each endotoxin contained one a factor which was not present in the other preparations

It was found that most anti Gc 8551 sera contained antibodies of very low titres to factor a_1 Only one of 5 different sera tested for agglutination of erythrocytes sensitized with determinant a of endotoxin VII (a_1) gave a titre of 128 whereas the titres of the remaining sera varied from 16 to 32 Anti Gc V serum contained no antibodies to factor a_3 Nevertheless both the absorption experiments and the inhibition tests have shown that endotoxin V possessed factor a_3

Distribution of the a *Factors and of Determinant* b *among Gonococci and Gram Negative Cocci of other Species*

Samples of 200 and 100 µg of crude aqueous ether endotoxin prepared from 24 strains of gonococci and 4 strains of meningococci were

TABLE 5

Minimal Inhibiting Dose (MID in µg) of Aqueous Ether Endotoxin from N gonorrhoeae Strains 8551 V and VII Determined by Inhibition of Haemagglutination with Unabsorbed and Absorbed Antisera

Test system made up of: Anti Gc	Determinant a of	MID of endotoxin: 8551	V	VII	Factor designation
VII unabs	End 8551	6 25	6 25	12 50	a_1
V abs end 8551	End V	>200	3 12	>200	a
VII abs end 8551	End V	>200	1 56	12 50	a_3
VII abs end V	End VII	>200	>200	12 50	a_4
8551 abs end V	End 8551	3 12	>200	>200	a
8551 abs end VII	End V	6 25	12 50	>200	a_6

Anti Gc Antiserum to whole gonococci

To test for cross reaction with bacteria other than gonococci and meningococci 16 agglutinating units of the various antisera were absorbed with 1 5 mg of lyophilized bacteria and then tested for agglutination of sensitized erythrocytes About 0 15 mg of lyophilized gonococci of the homologous strains was required for absorption of 16 units of these antibodies

None of the bacteria listed in Table 7 absorbed the antibodies to the factors a_1 a a_3 and a or the antibodies to determinant *b* *N canis* and *N denitrificans* absorbed the antibodies to a_4 and a_6 where is none of the other strains absorbed the antibodies to a_4 The antibodies to a_6 were also removed by absorption with *N catarrhalis* (one strain) *N pharyngis* (one strain) *N flavescens* (2 strains) and the *Veillonella* strain The results demonstrate some serological cross reactivity between the carbohydrate component of endotoxins from gonococci and apathogenic *Neisseria*

DISCUSSION

Aqueous ether endotoxin prepared from the reference gonococcal strains 8551 X and VII has been analysed and compared chemically and serologically The endotoxin preparations were essentially similar with respect to physical properties and chemical composition and proved to be complexes of lipid carbohydrate and protein While protein constituted from 80 to 90 per cent of the endotoxins the carbohydrate components composed of glucose galactose glucosamine and heptose amounted to less than 5 per cent With the reservation that minute amounts of other sugars may have escaped detection it is concluded that the endotoxins of the reference strains belong to the same chemotype *i e* have the same qualitative sugar composition

Determinant b

The protein determinant (*b*) of endotoxins prepared from the reference strains cross reacted serologically and haemagglutination inhibition and absorption experiments presented evidence that these determinants are identical Furthermore all the other strains of gonococci examined (64 strains) and of meningococci (4 strains) cross reacted with determinant *b* of endotoxin 8551 On the other hand strains of Gram negative cocci belonging to other species showed no cross reactivity These findings strongly indicate that determinant *b* is a group reactive antigen common to gonococci and meningococci Therefore tests for cross reactivity with determinant *b* may offer a supplementary method for differentiation of gonococci and meningococci on the one hand and other Gram negative cocci on the other

A close antigenic relationship between gonococci and meningococci has been demonstrated by several investigators *Wilson* (27) and *Deacon et al* (6) using bacterial agglutination and fluorescent anti

examined for inhibition of haemagglutination in the various test systems for a factors shown in Table 5 The preparations were also tested for inhibition of haemagglutination in the test system for determinant b of endotoxin 8551

It appears from Table 6 that all gonococcal strains contained at least two of the a factors Although the factors occurred in different combinations 22 out of the 27 gonococcal strains including the reference strains belonged to groups comprising from 2 to 6 strains carrying identical factors While all strains of gonococci possessed factor a_1 factor a_6 was also found in all strains except strain VII and another strain with identical a factors ($ba_{1\ 3\ 4}$) Four strains possessed the same factors as strain 8551 ($ba_{1,\ \ 6}$) and 6 strains the factors of strain X ($ba_{1\ \ 3\ 6}$)

The a factors were however not restricted to gonococci since groups B and C of the meningococcal strains examined contained factor a_1 and groups A and B of the meningococcal strains contained factor a_6

All endotoxin preparations from the strains listed in Table 6 including those from meningococci gave inhibition in the test system for determinant b Anti Gc 8551 0 1 ml diluted 1 16 was absorbed with approximately 200 mg of wet cells of 40 other strains of gonococci One absorption with each of these strains removed the antibodies to determinant b indicating identity or close similarity of the b determinants

The serological activity of the endotoxin preparations from meningococci and from 5 strains of gonococci was compared in the test systems for a factors and for determinant b The minimal inhibiting doses varied between 1 56 and 25 µg

TABLE 7

Distribution of the Factors a_1 and a_6 in Gram Negative Cocci other than Gonococci and Meningococci Determined by Absorption of Antisera with Bacterial Cells

Strains tested		Factors a	a
N catarrhalis	NCTC 3622	—	—
N catarrhalis	4103	—	+
N pharyngis	4590	—	—
N pharyngis	4591	—	+
N flavescens	8263	—	+
N flavescens	3191	—	+
N cuniculi	10297	—	—
N canis	10296	+	+
N caviae	10293	—	—
N denitrificans	10295	+	+
N haemolysans	10243	—	—
Mima polymorpha	7976	—	—
Herellea vaginalis	7844	—	—
Herellea vaginalis	7250	—	—
Veillonella		—	+

+ and — indicate presence or absence of the a factors

To test for cross reaction with bacteria other than gonococci and meningococci 16 agglutinating units of the various antisera were absorbed with 1.5 mg of lyophilized bacteria and then tested for agglutination of sensitized erythrocytes. About 0.15 mg of lyophilized gonococci of the homologous strains was required for absorption of 16 units of these antibodies.

None of the bacteria listed in Table 7 absorbed the antibodies to the factors a_1, a_2, a_3 and a_5 or the antibodies to determinant *b*. *N. canis* and *N. denitrificans* absorbed the antibodies to a_4 and a_6 whereas none of the other strains absorbed the antibodies to a_4. The antibodies to a_1 were also removed by absorption with *N. catarrhalis* (one strain), *N. pharyngis* (one strain), *N. flavescens* (2 strains) and the *Veillonella* strain. The results demonstrate some serological cross reactivity between the carbohydrate component of endotoxins from gonococci and apathogenic *Neisseria*.

DISCUSSION

Aqueous ether endotoxin prepared from the reference gonococcal strains 8551 V and VII has been analysed and compared chemically and serologically. The endotoxin preparations were essentially similar with respect to physical properties and chemical composition and proved to be complexes of lipid, carbohydrate and protein. While protein constituted from 80 to 90 per cent of the endotoxins, the carbohydrate components, composed of glucose, galactose, glucosamine and heptose, amounted to less than 5 per cent. With the reservation that minute amounts of other sugars may have escaped detection, it is concluded that the endotoxins of the reference strains belong to the same chemotype, i.e. have the same qualitative sugar composition.

Determinant b

The protein determinant (*b*) of endotoxins prepared from the reference strains cross reacted serologically and haemagglutination inhibition and absorption experiments presented evidence that these determinants are identical. Furthermore all the other strains of gonococci examined (64 strains) and of meningococci (4 strains) cross reacted with determinant *b* of endotoxin 8551. On the other hand strains of Gram negative cocci belonging to other species showed no cross reactivity. These findings strongly indicate that determinant *b* is a group reactive antigen common to gonococci and meningococci. Therefore tests for cross reactivity with determinant *b* may offer a supplementary method for differentiation of gonococci and meningococci on the one hand and other Gram negative cocci on the other.

A close antigenic relationship between gonococci and meningococci has been demonstrated by several investigators. *Wilson* (27) and *Deacon et al.* (6) using bacterial agglutination and fluorescent anti

body techniques respectively have shown that gonococci and meningococci share heat stable antigens Serological cross reactivity of toxic materials from gonococci and meningococci has been reported (1 2 23) *Reyn* (20) using the complement fixation test observed a thermostable common gonococcus antigen In view of the fact that determinant *b* is thermostable (16) and is closely associated with the endotoxin (17) it seems highly probable that this determinant is identical to or part of the cross reacting antigens described by these authors *Chanarin* (3) prepared an erythrocyte sensitizing antigen by extraction of gonococci with alkali and found that this antigen was type specific (type I and type II) In all 59 of 67 strains belonged to type I 8 strains to type II Meningococci cross reacted with the type I antigen It has previously been shown that erythrocytes sensitized with the alkali extract acquire the specificity of determinant *b* (16 19) a finding which indicates that determinant *b* is identical with *Chanarin*s type I antigen No type II antigen has been detected in this study

The author has previously reported that 55 per cent of blood donor sera contained antibodies to erythrocytes sensitized with the alkali extract (14) *i e* antibodies to determinant *b* Contact with meningococci may have induced the formation of the determinant *b* antibodies in many of the healthy individuals

Antigenic determinants of endotoxins (O antigens) are usually of carbohydrate nature (13) although determinants of protein nature have also been reported (10) However both the present study and results reported previously (14 15 16 17 19) have clearly shown that the protein component plays an important part in the serology of the endotoxin from gonococci

Determinant a

The *a* determinants from the reference strains cross reacted serologically but were not identical since the preparations failed to inhibit completely the haemagglutination in heterologous test systems for the *a* determinants The results of cross absorptions of antisera and of haemagglutination inhibition tests with cross absorbed sera (cfr Table 4 and 5) showed that each *a* determinant comprises several antigenic specificities (*a* factors) Six *a* factors (a_1 a_6) were revealed The cross reactions were thus due to *a* factors shared by the cross reacting endotoxins The possibility that some of the *a* factors consist of more than one antigenic specificity cannot be excluded Moreover other *a* factors may well exist within the species *N gonorrhoeae* in addition to the *a* factors described In order to settle these matters experiments with additional reference strains and antisera to these strains are required

As the effect of cross absorption with phenol water lipopolysaccharides or lyophilized bacteria was the same as that of cross absorp

tion with aqueous ether endotoxin the latter preparation must contain all the antigenic specificities which belong to the carbohydrate component of gonococcal endotoxins

The a factors of each strain of gonococci certainly belong to one and the same molecular complex analogous to the O factors of *Salmonella* endotoxins (13) It was found that the endotoxins prepared from the reference strains belonged to the same chemotype with regard to sugar constituents *Salmonella* and *Escherichia* O antigens belonging to the same chemotype may include several serotypes (13 30) *Luderitz et al* (13) suggested that in these instances the same sugars are linked in different ways which would explain the difference in specificity The results of tests for inhibition of haemagglutination with mono and disaccharides have indicated that the difference in specificity of the various a factors of gonococci can be explained in the same way As reported earlier galatose and lactose inhibited the reaction of anti Gc 8551 with determinant a of endotoxin 8551 (18) Investigations currently in progress have shown that galactose and lactose also inhibit the combination of anti Gc V with determinant a of endotoxin V but not the combination of anti Gc VII with determinant a of endotoxin VII Further investigations along these lines will probably provide valuable information about the structures which determine the specificity of the various a factors

The six a factors demonstrated in this study were found to be widely distributed among strains of gonococci Although the combination of factors varied considerably 22 out of 27 strains could be divided into 6 groups each group comprising from 2 to 6 strains with identical factors Classification of gonococci based on the a factors thus seems possible but further investigations are needed to evaluate the practical application of these findings All strains of gonococci examined contained factor a_1 which therefore may be common to all gonococci However this factor is not limited to the species *N gonorrhoeae* since 2 out of 4 strains of meningococci examined cross reacted with factor a_1 Moreover 2 strains of meningococci cross reacted with factor a_6 The close antigenic relationship between gonococci and meningococci is also apparent from these cross reactions

Some strains of Gram negative cocci other than gonococci and meningococci cross reacted with factor a factor a or both Accordingly some antigenic relationship between gonococci and apathogenic *Neisseria* species exists in agreement with observations made by others (4 5 6 23 24)

To my knowledge there are no published reports on multispecificity of the carbohydrate component of endotoxins from *Neisseria* species It is obvious that the endotoxin of gonococci is a highly complex substance which requires further investigations for its characterization

SUMMARY

Aqueous ether endotoxin prepared from three strains of gonococci contained from 80 to 90 per cent of protein and small amounts of carbohydrate and lipid. Each endotoxin contained glucose, galactose, glucosamine and heptose.

Tests for serological cross reactivity of the endotoxins presented evidence that the antigenic determinant of carbohydrate nature (*a*) was multispecific. Six antigenic specificities (*a* factors) were demonstrated. Endotoxin prepared from other strains of gonococci cross reacted with several of the *a* factors. Some strains of meningococci and other Gram negative cocci cross reacted with one or two of the *a* factors.

The antigenic determinant which belongs to the protein component (*b*) of the aqueous ether endotoxin was found to be a group reactive antigen common to gonococci and meningococci. Strains of Gram negative cocci belonging to other species gave no cross reaction with this determinant.

REFERENCES

1 *Boor A K & Miller C P* A study on bacterial proteins with special consideration of gonococcus and meningococcus J exp Med 59 63–74 1934
2 *Boor A K & Miller C P* A carbohydrate lipid fraction of gonococcus and meningococcus J infect Dis 75 47–57 1944
3 *Chanarin I* An investigation of Neisseria gonorrhoeae by a red cell sensitization technique J Hyg (Lond) 52 425–443 1954
4 *Danielsson D* The demonstration of N *gonorrhoeae* with the aid of fluorescent antibodies 1 Immunological studies of antigonococcal sera and their fluorescein labelled globulins with particular regard to specificity Acta derm venereol (Stockh) *43* 451–464 1963
5 *Danielsson D* The demonstration of N *gonorrhoeae* with the aid of fluorescent antibodies 4 Studies by immunofluorescence and double diffusion in gel technique on the antigenic relationship between N *gonorrhoeae* and other *Neisseria* strains Acta path microbiol scand *64* 267–276 1965
6 *Deacon W E, Peacock W L Jr, Freeman E M & Harris A* Identification of *Neisseria gonorrhoeae* by means of fluorescent antibodies Proc Soc exp Biol (N Y) *101* 322–325 1959
7 *Dische Z* Über einige neue charakteristische Farbreaktionen der Thymonukleinsäure und eine Mikromethode zur Bestimmung derselben in tierischen Organen mit Hilfe dieser Reaktionen Mikrochemie *8* 4–32 1930
8 *Dische Z* Qualitative and quantitative colorimetric determination of heptoses J biol Chem *204* 983–997 1953
9 *Fiske C H & Subbarow Y* The colorimetric determination of phosphorus J biol Chem *66* 375–400 1925
10 *Homma J Y & Suzuki N* The protein moiety of the endotoxin of *Pseudomonas aeruginosa* Ann N Y Acad Sci *133* 508–526 1966
11 *Kabat E A & Mayer M M* Experimental immunochemistry Thomas Springfield Ill 1961 pp 476–483
12 *Lowry O H, Rosebrough N J, Farr A L & Randall R J* Protein measurement with the Folin phenol reagent J biol Chem *193* 265–275 1951
13 *Lüderitz O, Staub A M & Westphal O* Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae* Bact Rev *30* 192–255 1966
14 *Mæland J A* Antibodies in human sera against antigens in gonococci demonstrated by a passive haemolysis test Acta path microbiol scand *67* 102–110 1966

15 *Mæland J A* Serological properties of antisera to *Neisseria gonorrhoeae* antigens Acta path microbiol scand *69* 145–155 1967

16 *Mæland J A* Antigenic properties of various preparations of *Neisseria gonorrhoeae* endotoxin Acta path microbiol scand *73* 413–422 1968

17 *Mæland J A* Antigenic determinants of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae* Acta path microbiol scand *76* 475–483 1969

18 *Mæland J A* Immunochemical characterization of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae* Acta path microbiol scand *76* 484–492 1969

19 *Mæland J A* Serological properties of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae* Acta path microbiol scand *77* 479–488 1969

20 *Reyn A* Serological studies on gonococci III Thermostability and biochemical nature of the gonococcus antigens Durability of the factor sera Discussion Acta path microbiol scand *26* 252–268 1949

21 *Rondle C J M & Morgan W T J* The determination of glucosamine and galactosamine Biochem J *61* 586–589 1955

22 *Snyder F & Stephens N* A simplified spectrophotometric determination of ester groups in lipids Biochim biophys Acta (Amst) *34* 244–245 1959

23 *Stokinger H E Ackerman H & Carpenter C M* Studies on the gonococcus II Properties of an antigenic fraction isolated from cell free gonococcal broth supernatants J Bact *47* 141–147 1944

24 *Stokinger H E Carpenter C M & Plack J* Studies on the gonococcus III Quantitative agglutinative reactions of the *Neisseria* with special reference to Neisseria gonorrhoeae J Bact *47* 149–157 1944

25 *Tauber H* Fatty acid ester groups in lipopolysaccharide endotoxins Fed Proc *19* 245 1960

26 *Trevelyan W E Procter D P & Harrison J S* Detection of sugars on paper chromatograms Nature (Lond) *166* 444–445 1950

27 *Wilson J F* A serological study of the meningococcus J Path Bact *72* 111–119 1956

28 *Winzler R J* Determination of serum glycoproteins In Glick D (Ed) Methods of biochemical analysis vol II Interscience Publishers Inc New York 1955 pp 279–311

29 *Youngburg G E & Youngburg M V* Phosphorus metabolism J Lab clin Med *16* 158–166 1930

30 *Ørskov F, Ørskov I Jann B Jann K Muller Seitz E & Westphal O* Immunochemistry of *Escherichia coli* O antigens Acta path microbiol scand *71* 339–358 1967

MATERIALS AND METHODS

Animals The mice were highly inbred female C_3H mice raised at our institute The rabbits were non inbred white rabbits

Antilymphocyte serum ALS was prepared from a rabbit by i v injection of 200 × 10^6 C_3H lymph node cells suspended in phosphate buffered saline (PBS) This was repeated three times at weekly intervals and one week after the last injection the animal was bled This serum was used for the preliminary experiment The rabbit was boosted and rebled twice and the serum from the second rebleeding was used in the experiments proper The sera were inactivated and kept at —20 C until required

Normal rabbit serum NRS was obtained in the same way except that the animal was injected with saline only

The sera were tested for haemagglutinins and leuco agglutinins against C_3H red and white cells and titres of 16 and 32 respectively were found in the first prepara tion of ALS while no such activity was found in the NRS

Virus The virus was a 10 per cent clarified homogenate of spleens harvested seven days after i p LCM infection of adult C_3H female mice

Virus titrations were carried out in ten fold dilutions in PBS of heparinized blood drawn from the retro orbital plexus Each dilution was injected into four ordinary Swiss mice The deaths occurring within 14 days were recorded and the titres cal culated according to *Karber* s method (6) The titres are expressed as log 10 LD / 0 03 cc

CF antibodies were measured as described in a previous report (16)

Donor mice Mice which have nursed LCM infected offspring develop a life long immunity to reinfection and persisting CF antibodies (8) The spleens of such immune animals were used for transplantation Before transplantation the sera of the mice were titrated for CF antibodies and titres between 64 and 512 were found

Recipient mice Mice which are infected with >10 LD_0 LCM virus i p within 18 hours of birth develop a life long tolerant infection (7) In this laboratory these virus carrier mice are characterized by a constant viraemia with titres ≥ 2 3 CF antibodies <4 and no signs of disease Female virus carrier mice of this type were used as recipients of the syngeneic donor cells Before transplantation the viraemia was >3 0 in all recipients and the CF antibodies <4

Transplantation of cells Spleens from ten immune donors were excised under aseptic precautions cut into pieces and pressed through a stainless steel mesh into Hank s BSS with penicillin and streptomycin After careful mixing the cells were divided into three parts one containing 1/2 and the remaining two 1/4 of the original volume They were then washed three times in Hanks BSS and resus pended in different fluids The first portion (1/2 of the cells) was resuspended in 6 ml of undiluted ALS and incubated at 37 C for 1½ hours with frequent mixing No appreciable macroscopic or microscopic agglutination occurred during the in cubation period Thereafter the cells were washed three times in Hanks BSS resus pended and counted after staining with eosin This revealed uptake of eosin by 35 per cent of the cells Finally 30 × 10^6 living cells were injected i p into the re cipient virus carrier mice

The second portion of the cells (1/4 of the original volume) was treated identic ally *except that they were incubated with 3 ml of undiluted NRS The counting this* time revealed uptake of eosin by 25 per cent of the cells

Th third portion containing the remaining 1/4 of the original immune cells was incubated with Hanks BSS and here the eosin uptake was 30 per cent

The percentage of cells which took up eosin in the three portions of cells was higher than is usually seen in our transplantation experiments (10–15 per cent) This was thought to be due to the relatively long in vitro processing

B th the second and the third part of cells were injected into virus carrier re cipients in doses of 30 × 10^6 living cells per mouse

In all three groups of recipients the virus contents in the blood and the CF antibodies in the sera were measured individually at intervals

RESULTS

In a preliminary experiment a group of five LCM virus carrier mice was transplanted i p with 16 × 10 living ALS treated syngeneic im

Acta path microbiol scand 77 518–526 1969

The Institute of Medical Microbiology University of Copenhagen Denmark

EFFECT OF ANTILYMPHOCYTE SERUM ON ADOPTIVE IMMUNIZATION OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS CARRIER MICE

By

CLAUS LUNDSTEDT

Received 14 v 69

The marked suppressive effect of antilymphocyte serum (ALS) on cell mediated immunity has been demonstrated several times recently (10 11 18) This is in contrast to the almost complete absence of effect on the humoral antibody response especially on the secondary response (3 5) In vitro ALS is capable of exerting a complement dependent lymphocytotoxic effect or—in the absence of complement—a leuco agglutinating effect However these effects cannot always be related to the in vivo effect on for example skin grafts (4 9)

During the past few years evidence has been accumulating which indicates that the virus eliminating mechanism seems to be cell mediated in a least some virus infections (1 2 3 7 12 17) Thus it has been shown that adoptive immunization of tolerant lymphocytic choriomeningitis (LCM) virus carrier mice can be accomplished only by immunologically competent cells (normal or immune) and not by hyperimmune anti LCM serum (15) Furthermore in this laboratory we have been able to provoke a viraemia in LCM immune mice by a long ALS treatment without significantly affecting the content of complement fixing (CF) antibodies in the sera of these mice (17) The viraemia was thought to arise from the observed virus reservoir in the kidneys thymuses or lungs of such immune animals (14) Moreover rubella infected children have been shown to harbour virus in their naso pharynx for months whilst at the same time the serum contains neutralizing fluorescent haemagglutination inhibiting and CF antibodies (12)

The present experiments were therefore undertaken to throw further light upon the relative importance of cell mediated and humoral immunity in LCM infection The report describes the effect of ALS on the ability of syngeneic LCM immune spleen cells to confer adoptive immunization on LCM tolerant virus carrier mice

---

Requests for reprints should be addressed to Dr Claus Lundstedt 22 Juliane Maries Vej DK 2100 Copenhagen Ø Denmark

This study has been supported by grants from the *Danish Cancer Society* and the *Einer Willumsen s Memorial Fund*

MATERIALS AND METHODS

Animals The mice were highly inbred female C_3H mice raised at our institute The rabbits were non inbred white rabbits

Antilymphocyte serum ALS was prepared from a rabbit by i v injection of 200 × 10^6 C_3H lymph node cells suspended in phosphate buffered saline (PBS) This was repeated three times at weekly intervals and one week after the last injection the animal was bled This serum was used for the preliminary experiment The rabbit was boosted and rebled twice and the serum from the second rebleeding was used in the experiments proper The sera were inactivated and kept at −20 C until required

Normal rabbit serum NRS was obtained in the same way except that the animal was injected with saline only

The sera were tested for haemagglutinins and leuco agglutinins against C_3H red and white cells and titres of 16 and 32 respectively were found in the first preparation of ALS while no such activity was found in the NRS

Virus The virus was a 10 per cent clarified homogenate of spleens harvested seven days after i p LCM infection of adult C H female mice

Virus titrations were carried out in ten fold dilutions in PBS of heparinized blood drawn from the retro orbital plexus Each dilution was injected into four ordinary Swiss mice The deaths occurring within 14 days were recorded and the titres calculated according to *Kärber's* method (6) The titres are expressed as log 10 LD_{50}/ 0 03 cc

CF antibodies were measured as described in a previous report (16)

Donor mice Mice which have nursed LCM infected offspring develop a life long immunity to reinfection and persisting CF antibodies (8) The spleens of such immune animals were used for transplantation Before transplantation the sera of the mice were titrated for CF antibodies and titres between 64 and 512 were found

Recipient mice Mice which are infected with $>10\ LD_{50}$ LCM virus i p within 18 hours of birth develop a life long tolerant infection (7) In this laboratory these virus carrier mice are characterized by a constant viraemia with titres $\geq 2\ 3$ CF antibodies <4 and no signs of disease Female virus carrier mice of this type were used as recipients of the syngeneic donor cells Before transplantation the viraemia was $>3\ 0$ in all recipients and the CF antibodies <4

Transplantation of cells Spleens from ten immune donors were excised under aseptic precautions cut into pieces and pressed through a stainless steel mesh into Hanks BSS with penicillin and streptomycin After careful mixing the cells were divided into three parts one containing 1/2 and the remaining two 1/4 of the original volume They were then washed three times in Hanks BSS and resuspended in different fluids *The first portion (1/2 of the cells)* was resuspended in 6 ml of undiluted ALS and incubated at 37 C for 1½ hours with frequent mixing No appreciable macroscopic or microscopic agglutination occurred during the *incubation period* Thereafter the cells were washed three times in Hanks BSS resuspended and counted after staining with eosin This revealed uptake of eosin by 35 per cent of the cells *Finally 30 × 10^6* living cells were injected i p into the recipient virus carrier mice

The second portion of the cells (1/4 of the original volume) was treated identically except that they were incubated with 3 ml of undiluted NRS The counting this time revealed uptake of eosin by 25 *per cent of the* cells

The third portion containing the remaining 1/4 of the original immune cells was incubated with Hanks BSS *and here the eosin* uptake was 30 per cent

The percentage of cells which took up eosin in the three portions of cells was higher than is usually seen in our *transplantation* experiments (10–15 per cent) This was thought to be due to the relatively long in vitro processing

Both the second and the third part of cells were injected into virus carrier recipients in doses of 30 × 10^6 living cells per mouse

In all three *groups of* recipients the virus contents in the blood and the CF antibodies in the sera were measured individually at intervals

RESULTS

In a preliminary experiment a group of five LCM virus carrier mice was transplanted i p with 16 × 10^6 *living* ALS treated syngeneic im

mune spleen cells isolated from four immune donors The cells had been incubated with 1 5 ml of ALS and washed as described

One mouse in the group died 12 days after transplantation and was never investigated A second mouse died after 40 days At autopsy both of these mice were found to have a thymoma A third mouse died after four months without any obvious reason but it must be noted that all the mice in this group were 8–9 months old at the time of transplantation

The four mice which were investigated all developed CF antibodies in titres initially varying between 512 and 2048 In the two long term survivors the titres gradually decreased to about 128 in the course of 2–3 months However the virus titres remained at virus carrier levels (i e ≥2 3) throughout the observation period which was 253 days in the case of mice surviving for the longest period

Mice in one control group comprising five LCM virus carrier mice received 16 × 16[6] living cells from the same pool of cells However these cells were treated with 1 5 ml of NRS In this group all the mice developed CF antibodies with titres between 1024 and 8192 stabilizing at about 1024 in 1–2 months The virus titres gradually declined in 1–2 months to titres ≤ 0 5 Thus a complete adoptive immunization was obtained One mouse died 78 days after transplantation

A second control group also consisted of five LCM tolerant animals which received 16 × 10^6 living cells from the same pool of cells as the experimental group But here the cells were incubated with Hanks BSS instead of ALS or NRS All five mice developed CF antibodies in titres between 2048 and 16 384 decreasing in 1–2 months to about 1024 In three of the mice the virus was eliminated from the blood to titres ≤ 0 5 and a complete adoptive immunization was obtained The remaining two however retained their viraemia on virus carrier level as in the experimental group This to some extent invalidated the results an it was thought to be due to the dose of transplanted cells (16 × 10^6) which might have been near the lower limit of that necessary to obtain a constant effect

The experiments were therefore repeated with the procedure as described in materials and methods The important alterations from the preliminary experiments were 1) the transplanted dose of cells was now 30 × 10^6 and 2) the recipient animals were only about three months old at the time of transplantation The results are shown in Table 1 and Figs 1 and 2

The experimental group consisted of ten female virus carrier mice which received ALS treated syngeneic LCM immune spleen cel

As can be seen from Table 1 the results can be divided into groups Either the recipients developed CF antibodies (with ration in titres) and no major alteration in virus titres r adoptive immunization (seven mice nos 2 3 5 6 7 8 9) veloped CF antibodies which remained at a higher level f

TABLE 1

LCM Virus and CF Titre in Blood of LCM Virus Carrier Mice Transplanted with LCM Immune Syngeneic Cells

Animal groups	0 Virus	0 CF	15 Virus	15 CF	41 Virus	41 CF	77 Virus	77 CF	113 Virus	113 CF	166 Virus	166 CF
	Days after transplantation											
Experimental group receiving ALS treated cells												
Mouse no 1	3 0	<4	2 5	512	1 5	256	≤ 0 5	256	1 0	256	≤ 0 5	512
2	N D	N D	3 5	16	3 0	8	3 5	4	3 0	4	3 5	<4
3	3 3	<4	2 0	256	3 5	128	3 8	32	3 5	32	2 0	64
4	3 0	<4	3 8	≥ 2048	2 5	1024	≤ 0 5	512	≤ 0 5	512	1 0	512
5	3 0	<4	2 5	512	3 3	8	4 0	4	2 8	<4	3 3	<4
6	3 0	<4	3 5	1024	3 3	32	3 5	16	3 3	16	4 0	16
7	3 0	<4	2 5	≥ 2048	2 5	≥ 2048	1 8	512	3 3	64	4 0	32
8	≥ 3 5	<4	2 8	512	2 5	128	3 3	64	4 3	64	3 0	32
9	3 3	<4	3 3	1024	2 3	512	2 0	1024	1 5	512	3 3	512
10	≥ 3 5	<4	2 5	≥ 2048	1 3	1024	≤ 0 5	512	≤ 0 5	1024	≤ 0 5	≥ 2048
	3 3	<4										
Control group receiving NRS treated cells	5/5	5/5	4/4	4/4	3/3	3/3	3/3	2/3	3/3	2/3	2/3	2/3
	≥ 3 0	<4	≤ 0 5	≥ 2048	≤ 0 5	≥ 2048	≤ 0 5	512	≤ 0 5	≥ 2048	≤ 0 5	≥ 2048
Control group receiving Hanks BSS treated cells	5/5	5/5	4/4	3/4†	4/4	4/4	4/4	2/4	4/4	2/4	3/4§	2/4
	≥ 3 3	<4	≤ 0 5	≥ 2048	≤ 0 5	≥ 2048	≤ 0 5	≥ 2048	≤ 0 5	512	≤ 0 5	≥ 2048

N D = Not Done   Remaining mouse ≥ 2048   Remaining mouse/mice 1024   Remaining mouse 1 0   † Remaining mouse Not done   § Remaining mouse 2 0

mune spleen cells isolated from four immune donors The cells had been incubated with 1 5 ml of ALS and washed as described

One mouse in the group died 12 days after transplantation and was never investigated A second mouse died after 40 days At autopsy both of these mice were found to have a thymoma A third mouse died after four months without any obvious reason but it must be noted that all the mice in this group were 8-9 months old at the time of transplantation

The four mice which were investigated all developed CF antibodies in titres initially varying between 512 and 2048 In the two long term survivors the titres gradually decreased to about 128 in the course of 2-3 months However the virus titres remained at virus carrier levels (i e ≥ 2 3) throughout the observation period which was 253 days in the case of mice surviving for the longest period

Mice in one control group comprising five LCM virus carrier mice received 16 $\times$ 16^6 living cells from the same pool of cells However these cells were treated with 1 5 ml of NRS In this group all the mice developed CF antibodies with titres between 1024 and 8192 stabilizing at about 1024 in 1-2 months The virus titres gradually declined in 1-2 months to titres $\leq$ 0 5 Thus a complete adoptive immunization was obtained One mouse died 78 days after transplantation

A second control group also consisted of five LCM tolerant animals which received 16 $\times$ 10^6 living cells from the same pool of cells as the experimental group But here the cells were incubated with Hanks BSS instead of ALS or NRS All five mice developed CF antibodies in titres between 2048 and 16 384 decreasing in 1 2 months to about 1024 In three of the mice the virus was eliminated from the blood to titres $\leq$ 0 5 and a complete adoptive immunization was obtained The remaining two however retained their viraemia on virus carrier level as in the experimental group This to some extent invalidated the results an it was thought to be due to the dose of transplanted cells (16 $\times$ 10^6) which might have been near the lower limit of that necessary to obtain a constant effect

The experiments were therefore repeated with the procedure as described in materials and methods The important alterations from the preliminary experiments were 1) the transplanted dose of cells was now 30 $\times$ 10^6 and 2) the recipient animals were only about three months old at the time of transplantation The results are shown in Table 1 and Figs 1 and 2

The experimental group consisted of ten female virus carrier mice which received ALS treated syngeneic LCM immune spleen cells i p As can be seen from Table 1 the results can be divided into two distinct groups Either the recipients developed CF antibodies (with great variation in titres) and no major alteration in virus titres i e a partial adoptive immunization (seven mice nos 2 3 5 6 7 8 9) or they developed CF antibodies which remained at a higher level for a longer

TABLE 1

LCM Virus and CF Titers in Blood of LCM Virus Carrier Mice Transplanted with LCM Immune Syngeneic Cells

Animal groups	Days after transplantation											
	0		15		41		77		113		160	
	Virus	CF	Virus	CF	Virus	CF	Virus	CF	Virus	CF	Virus	CF
Experimental group receiving ALS treated cells												
Mouse no 1	3.0	<4	2.5	512	1.5	256	≤ 0.5	256	1.0	256	≤ 0.5	512
2	ND	ND	3.5	16	3.0	8	3.5	4	3.0	4	3.5	<4
3	3.3	<4	2.0	256	3.5	128	3.8	32	3.5	32	2.0	64
4	3.0	<4	3.8	≥ 2048	2.5	1024	≤ 0.5	512	≤ 0.5	512	1.0	512
5	3.0	<4	2.5	512	3.3	8	4.0	4	2.8	<4	3.3	<4
6	3.0	<4	3.5	1024	3.3	32	3.5	16	3.3	16	4.0	16
7	≥ 3.5	<4	2.5	≥ 2048	2.5	≥ 2048	1.8	512	3.3	64	4.0	32
8	3.3	<4	2.8	512	2.5	128	3.3	64	4.3	64	3.0	32
9	≥ 3.5	<4	3.3	1024	2.3	512	2.0	1024	1.5	512	3.3	512
10	3.3	<4	2.5	≥ 2048	1.3	1024	≤ 0.5	512	≤ 0.5	1024	≤ 0.5	≥ 2048
Control group receiving NRS treated cells	5/5 ≥ 3.0	5/5 <4	4/4 ≤ 0.5	4/4 ≥ 2048	3/3 ≤ 0.5	3/3 ≥ 2048	3/3 ≤ 0.5	2/3 512	3/3 ≤ 0.5	2/3 ≥ 2048	2/3 ≤ 0.5	2/3 ≥ 2048
Control group receiving Hanks BSS treated cells	5/5 ≥ 3.3	5/5 <4	4/4 ≤ 0.5	3/4† ≥ 2048	4/4 ≤ 0.5	4/4 ≥ 2048	4/4 ≤ 0.5	2/4 ≥ 2048	4/4 ≤ 0.5	2/4 512	3/4§ ≤ 0.5	2/4 ≥ 2048

ND = Not Done   Remaining mouse ≥ 2048   Remaining mouse/mice 1024   Remaining mouse 1.0   † Remaining mouse Not done   § Remaining mouse 2.0

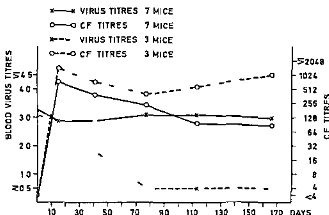


Fig 1

The effect of transplantation of immune ALS treated lymphoid cells to ten syngeneic virus carrier recipients The two alternative courses are shown For further explanation see text

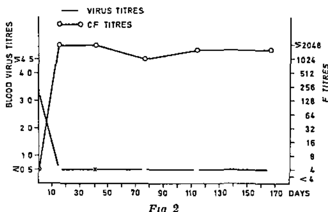


Fig 2

The effect of transplantation of immune NRS treated lymphoid cells to five syngeneic virus carrier recipients

period than in the mice just mentioned (three mice nos 1 4 10) But in these three mice the blood virus content gradually diminished and disappeared within about two months of the transplantation (*i e* a complete adoptive immunization) Up to the present time the mice have been observed for 166 days and CF and virustitrations are still performed at intervals to discover possible late variations from the course described

The three mice in the experimental group which were completely immunized differed in two respects both from the remainder of the experimental group and from the controls In comparison to the seven partially immunized animals in the experimental group these three mice showed characteristically higher levels of CF antibodies during the whole observation period after transplantation and in comparison

to the controls in which the blood virus was rapidly eliminated these three mice showed a more gradual decrease of the blood virus content over a period of ten weeks

One control group consisted of five female virus carrier mice which were treated with NRS incubated spleen cells from the same donors as those used for the experimental group All five mice were completely adoptively immunized within 15 days after transplantation with virus titres ≤ 0 5 and CF titres ≥ 2048 All five mice became ill about ten days after transplantation and two died four weeks after transplantation The remaining three recovered slowly but completely and after 166 days observation they were still immune

A second control group also consisted of five female virus carrier mice which were transplanted with Hanks BSS incubated spleen cells from the same donors as those used in the group of experimental animals and the first control group Four of these were also completely immunized within 15 days after transplantation with virus titres ≤ 0 5 and CF titres ≥ 2048 The fifth died before any virus of CF titrations were performed

Fig 2 shows the NRS treated controls The course of the Hanks BSS treated animals is practically identical to Fig 2 and is therefore not shown in a separate figure

DISCUSSION

If adult LCM virus carrier mice are adoptively immunized by transplantation of 30 × 10^6 syngeneic LCM immune spleen cells the normal series of events is rapid elimination of virus from the blood associated with very high titres of CF antibodies in the sera in 100 per cent of the animals (16)

The precent experiments however have shown that incubation of the lymphoid cells with ALS prior to transplantation modifies the usual course resulting in a state of partial tolerance (or immunity) characterized by persistent high virus and CF titres in the blood of the animals

Between the 10th and the 30th day after transplantation several animals displayed signs of illness with ruffled pelts hypokinesia and conjunctivitis most prominent in the ALS and NRS treated groups This was rather surprising since nothing similar has been observed in hundreds of adoptive immunizations carried out in this laboratory The reason for this might be either a more vigorous immunological conflict in partially immunized animals or else the simultaneous stimulation with other antigens (ALS or NRS)

It was demonstrated that the effect of ALS *in vitro* on LCM immune syngeneic spleen cells was to neutralize their ability (in seven out of ten animals) to eliminate the LCM virus from the blood of tolerant animals The lower CF antibody titre of these animals as compared

with the controls might not be a direct effect of ALS on the humoral antibody formation but could be explained by the persisting high virus level in these animals This virus could combine with a certain amount of the CF antibodies resulting in smaller measurable titres

It would thus appear that the effect of ALS on LCM immunity is primarily or exclusively directed against the virus eliminating component of the immune response with little or no effect on the antibody formation component of LCM immunity Other ALS experiments have demonstrated that ALS acts primarily or exclusively on cell mediated immunities (10 11) and this therefore adds further support to the accumulating evidence that the virus eliminating function in LCM infection and certain other virus diseases is cell mediated

Apparently there are at least two different populations of LCM reactive cells in the animals one which produces humoral antibodies and on which ALS has little or no effect and one which is responsible for the virus eliminating mechanism and which is sensitive to ALS treatment It is likely that this latter population represents a thymus dependent cell mediated immunity

It is noteworthy that the ALS treatment acted so strongly on the presensitized spleen cells that eradication of the cell mediated virus eliminating mechanism was complete in most of the animals This has also been shown by treatment in vivo of immune animals where a long course of ALS could provoke a new viraemia (17) And it is in line with other findings that ALS may suppress or completely eradicate previously established delayed hypersensitivities to a number of antigens including mumps in animals and man (13)

Nothing certain is known about the way in which the effects of ALS are brought about but one possibility is that the cells responsible for the cell mediated immunities are coated with ALS and that on injection into the animals with resultant contact with complement they might lyse

However in continuation of these experiments it remains to be demonstrated whether the same effect can be obtained by treating either the donors or the recipients in vivo before transplantation Such experiments are in progress

In conclusion it would therefore seem very probable that the immune response which leads to the elimination of LCM virus is brought about by the thymus dependent lymphocytes while the CF antibodies produced by the gut dependent lymphocytes – only represent a side effect of animal or no importance for the pathogenesis The same state of affairs is thought to govern the immunity to human measles infection (1)

SUMMARY

The effect of ALS on the ability of sensitized lymphoid cells to confer immunity on tolerant virus carrier mice was investigated LCM im

mune spleen cells were incubated in vitro with ALS and thereafter transplanted into syngeneic LCM tolerant mice These cells were not able to reduce the viraemia in the recipients (7/10 animals) but their ability to produce humoral antibodies was not affected thus leading to a split tolerance in these mice It was argued that the virus eliminating mechanism in LCM immunity (and probably in certain other viral infections) is cell mediated and selectively sensitive to ALS treatment while the production of humoral antibodies (complement fixing) cannot be inhibited Such antibodies are probably of little or no importance in the pathogenesis of LCM disease

REFERENCES

1 *Burnet F M* Measles as an index of immunological function Lancet 2 610–613 1968

2 *Hirsch M S & Murphy F A* Effect of antilymphoid sera on viral infections Lancet 2 37–40 1968

3 *Hirsch M S Nahmias A J Murphy F A & Kramer J H* Cellular immunity in Vaccinia infection of mice Anti thymocyte serum effects on primary and secondary responsiveness J exp Med *128* 121–132 1968

4 *Iwasaki Y Porter K A Amend J A Marchioro T L Zühlke V & Starzl T E* The preparation and testing of horse antidog and antihuman antilymphoid plasma or serum and its protein fractions Surg Gynec Obstet *124* 1–24 1967

5 *James K & Anderson N F* Effect of anti rat lymphocyte antibody on humoral antibody Nature (Lond) *213* 1195–1197 1967

6 *Karber G* Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche Naunyn Schmiedebergs Arch exp Path Pharmak *162* 480–483 1931

7 *Larsen J Hannover* Den lymphocytære choriomeningitis virusinfektion hos musen. Thesis Copenhagen 1968

8 *Larsen J Hannover* Development of humoral and cell mediated immunity to lymphocytic choriomeningitis virus in the mouse J Immunol *102* 941–946 1969

9 *Levey R H & Medawar P B* Some experiments on the action of antilymphoid antisera Ann N Y Acad Sci *129* 164–177 1966

10 *Levey R H & Medawar P B* The mode of action of antilymphocytic serum In Wolstenholme G E W & O Connor M (Eds) Antilymphocytic serum Ciba Foundation Study Group 29 J & A Churchill Ltd London 1967 p 72

11 *Monaco A P Wood M L Werf B A van der & Russell P S* Effect of antilymphocyte serum in mice dogs and man In Wolstenholme G E W & O Connor M (Eds) Antilymphocytic serum Ciba Foundation Study Group 29 J & A Churchill Ltd London 1967 p 111

12 *Sever J & White L R* Intrauterine viral infections Ann Rev Med *19* 471–486 1968

13 *Starzl T E Porter K A Iwasaki Y Marchioro T L & Kashiwagi N* The use of heterologous antilymphocyte globulin in human renal homotransplantation In Wolstenholme G E W & O Connor M (Eds) Antilymphocytic serum Ciba Foundation Study Group 29 J & A Churchill Ltd London 1967 p 4

14 *Volkert M & Larsen J Hannover* Studies on immunological tolerance to LCM virus 3 Duration and maximal effect of adoptive immunization of virus carriers Acta path microbiol scand *60* 577–587 1964

15 *Volkert M & Larsen J Hannover* Studies on immunological tolerance to LCM virus. 6 Immunity conferred on tolerant mice by immune serum and by grafts of homologous lymphoid cells Acta path microbiol scand *63* 172–180 1965

16 Volkert M Larsen J Hannover & Pfau C J Studies on immunological tolerance to LCM virus 4 The question of immunity in adoptively immunized virus carriers Acta path microbiol scand 61 268–282 1964
17 Volkert M & Lundstedt C The provocation of latent lymphocytic choriomeningitis virus infections in mice by treatment with antilymphocytic serum J exp Med 127 327–339 1968
18 Waksman B H Arbuoys S & Arnason B G The use of specific lymphocyte" antisera to inhibit hypersensitive reactions of the delayed type J exp Med 114 997–1021 1961

Acta path microbiol scand 77 527-541 1969

The Institute of Clinical Bacteriology (Head S Winblad MD)
University of Lund General Hospital Malmo Sweden

ELECTRON MICROSCOPIC STUDIES ON FLAGELLATION IN DIFFERENT STRAINS OF *YERSINIA ENTEROCOLITICA*

By

BIRGITTA NILÉHN

Received 18 iv 69

Bacteria referred to the new species *Yersinia enterocolitica* (*van Loghem* 1944 1946 *Frederiksen* 1964) have been described by various authors (review *Jacober* 1968) as non motile at 37 C but motile at temperatures below 28 C *Frederiksen* (1964) reported the occurrence of peritrichous flagella as a characteristic of the strains studied by him Comparative studies (*Nilehn* unpublished observations) of a large number of strains of *Yersinia enterocolitica* of various origins suggested however that non motile or poorly motile variants occur especially often among strains isolated from man dog and pig (O antigen type 3 *Winblad* 1967) as well as among certain biotypes isolated from hare while other hitherto known serological and biochemical types proved to be very motile at temperatures of about 25 C

These observations prompted a comparative investigation of the flagellation of different *Yersinia enterocolitica* strains grown in non defined complete media The *Yersinia enterocolitica* strains were selected in such a way as reasonably to represent different hitherto known serological or biochemical variants This paper is concerned with an electron microscopic examination of the flagellation in strains cultured under uniform conditions at 25 C and with a comparative study of the flagellation in strains cultured at 25 C and 37 C respectively

MATERIAL AND METHODS

Bacterial Strains

The strains used in the study were selected so as to represent the hitherto known antigenic groups (*Winblad* 1967 1968) and different biotypes such as indole positive (ind) strains of mixed origin (*Schleifstein & Coleman* 1939) indole negative

The study was supported by grants from the *Medical Faculty of the University of Lund* Sweden and from the *Swedish Medical Research Council*

I thank the staff of the Electron Microscope Laboratory Institute of Zoology University of Lund and especially Fil lic *Claes von Mecklenburg* for advice and kind help with the electron microscopy Mrs *E a Carls n* and Mrs *Gertrud Hansson* are thanked for skillful technical assistance

16 *Volkert M Larsen J Hannover & Pfau C J* Studies on immunological tolerance to LCM virus 4 The question of immunity in adoptively immunized virus carriers Acta path microbiol scand *61* 268–282 1964
17 *Volkert M & Lundstedt C* The provocation of latent lymphocytic choriomeningitis virus infections in mice by treatment with antilymphocytic serum J exp Med *127* 327–339 1968
18 *Walsman H B Arbuoys S & Arnason B C* The use of specific lymphocyte antisera to inhibit hypersensitive reactions of the delayed type J exp Med *114* 997–1021 1961

was then resuspended in sterile saline For electron microscopy negative staining with potassium phosphotungstate (PTA) pH 7 0 and preparation of grids were done according to *Hoeniger's* (1965) modification of the technique of *Thornley & Horne* (1962) Phosphotungstic acid was obtained from Riedel De Haen AG Selze Hann over bovine serum albumin used Protein Standard (Kabi AB Stockholm Sweden)

Non formalinized culture Preparations After 3 U tube passages at 25 C bacteria were cultured over night in BEB at 25 C and 37 C respectively A platinum loop ful of each broth culture was then subcultured each loopful in 100 ml of fresh broth The new cultures were maintained at 25 C and 37 C in water baths during continuous shaking For electron microscopy 5 ml samples were taken the cells were washed once with re distilled water and then negatively stained with PTA as described above and used for grid preparations Controls with grid preparations made directly from negatively stained broth cultures were also examined so as to ensure that the flagella were not affected by handling of the cells

The motility of the original over night broth cultures was studied on BEB agar plates at the respective temperatures In some cases cells gently harvested from these plates in sterile re distilled water were examined under the electron microscope

A few poorly motile strains were investigated repeatedly and after different lengths of incubation in order to find motile cells

Investigation of flagellated cultures after temperature shift from 25 C to 37 C
U tube passed cells of the strains Bojsen Møller 70 Becht 51 and MY 79b were used in the tests Each strain was inoculated into 100 ml of BEB previously passed through a 1 0 μ membrane filter (Sartorius Membrane Filter Type SM 11003 34/Göttingen Deutschland) The broth culture was incubated over night at 25 C during continuous shaking in a water bath and to a cell density of about 10^8–10^9/ml (viable counts) Half of the culture was then added to 250 ml of fresh BEB 25 C while the other half was transferred to 250 ml of fresh BEB prewarmed to 37 C. The cultures were then incubated during shaking at the respective temperatures At intervals growth was determined by viable counts and measurements of dry weight The pH during growth was continuously registered throughout the observation period of 6 hr For viable counts 0 1 ml samples were collected serially diluted 0 1 ml volumes were spread on human blood agar plates with glass beads and counted after 48 hr at 37 C Five fold plate series from each dilution were counted Dry weigths were determined in 10 ml samples centrifuged at 4 C 2000 g for 45 minutes washed once with re distilled water re centrifuged in the same way re suspended in 0 5 ml of re distilled water and dried for 180 minutes at 80 C.

Bacterial flagella were examined in the electron microscope after negative staining with PTA For staining 1 ml samples of broth culture were mixed with an equal volume of a 2 per cent PTA solution pH 7 0 The approximate number of flagellated cells (per cent) and the average number of flagella per cell (total number of cells) were calculated from counts of at least 200 cells from each preparation

Electron microscopy Formvar and carbon coated copper grids prepared in the way described above were examined in an electron microscope Hitachi HS 70 The proportion of flagellated cells and the number of flagella per cell were determined directly during microscopy The wavelengths (WL) the amplitudes (A) and the lengths of individual flagella were measured from electron micrographs The *formula* given by *Leifson et al* (1955) was used for calculating the spiral unit lengths (SUL) of the flagella

RESULTS

Flagellation at 25 C

Peritrichous flagella apparently randomly distributed from the bacterial soma could be demonstrated in all of the types of *Yersinia enterocolitica* studied here

Quantitatively however the flagellation differed from one group of strains to another The percentage of flagellated cells in the different strains and maximum number of flagella observed per cell in 48 hr batch cultures at 25 C are given in Table 1 It is clear from the table

(ind) xylose positive (xyl) strains of chinchilla or human origin (Hermans & Terpstra 1963 *Daniels & Goudzwaard* 1963 *Nilehn* 1967a b *Nilehn et al* 1968) ind xyl strains isolated from man dog or pig (*Hassig et al* 1949 *Becht* 1962 *Dickinson & Mocquot* 1961 *Nilehn* 1967 a b) and NO_3 non reducing strains (NO_3) isolated from hares (*Mollaret & Lucas* 1965)

Strains Used

Strain no	Origin	O antigen type (*Winblad* 1967 1968)	Biotype
1) Becht 51	Chinchilla	1	ind xyl
2) Daniels 924	Chinchilla	2	ind xyl
3) Daniels 1028	Hare	2	NO_3
4) Lucas 404	Hare	2	NO_3
5) Dickinson 07	Pig	3	ind xyl
6) MY 0– Winblad	Man	3	ind xyl
7) MY 2	Man	3	ind xyl
8) MY 57	Man	3	ind xyl
9) MY 60	Man	3	ind xyl
10) MY 134	Man	3	ind xyl
11) Becht Hund 200	Dog	3	ind xyl
12) B Knox 1017/60 61	Chinchilla	4	ind xyl
13) Ye 123–Vache	Cow	5	ind xyl
14) Bojsen Møller 70	Man	6	ind xyl
15) Borg Petersen SP 6613	Guinea pig	7	ind xyl
16) Albany 33114	Man	8	ind xyl
17) Albany 5819	Man	8	ind xyl
18) MY 39	Man	9	ind xyl
19) MY 79b	Man	9	ind xyl

Strains were kindly placed at our disposal by Prof E Thal The State Veterinary Medical Institute Stockholm (strains 1 2 and 11 = Thal 348 338 356) by Dr W Frederiksen The State Serum Institute Regional Department Aalborg (strains 3 4 5 12 14 15 16 17 = P 372 368 253 76 219 413 310 311) and by Prof H H Mollaret Institut Pasteur Paris (strain 13) Strains 6 7 8 9 10 18 19 from Malmo *Yersinia enterocolitica* collection

Media

Tryptose glucose broth (TGB) Bacto Tryptose (B 124 Difco) 20 g glucose 2 g NaCl 5 g Na_2HPO_4 $2H_2O$ 2.5 g aq dest 1000 ml Autoclaved for 15 minutes at 120°C pH 7.2

Beef extract broth (BEB) Beef extract (B 126 Difco) 5 g Proteose peptone no 3 (B 122 Difco) 10 g NaCl 3 g Na_2HPO_4 $2H_2O$ 2 g aq re dest 1000 ml Autoclaved for 30 minutes at 120°C pH 7.1

Semi solid agar for motility tests (BEB agar) BEB Bacto agar (B 140 Difco) 0.5 per cent Poured into plastic Petri dishes 9 cm in diameter 20 ml/dish

Methods

Strains to be investigated were taken from lyophilized cultures or from stock cultures (+4°C) in one case (MY 134) a fresh isolate was used

Formalinized culture preparations The strains were cultured on human blood agar at 25°C for 48 hr After 1 tube passage at 25°C (BEB agar) cells were inoculated with a platinum loop into a 200 ml volume of TGB in a 500 ml Erlenmeyer retort The culture was then cautiously shaken for 48 hr at 25°C in a water bath after which 0.5 ml of concentrated formalin was added followed by continued shaking for another 24 hr Centrifuged at 1060 g for 30 minutes The deposit

was then resuspended in sterile saline For electron microscopy negative staining with potassium phosphotungstate (PTA) pH 7 0 and preparation of grids were done according to *Hoeniger's* (1965) modification of the technique of *Thornley & Horne* (1962) Phosphotungstic acid was obtained from Riedel De Haen AG Selze Hann over bovine serum albumin used Protein Standard (Kabi AB Stockholm Sweden)

Non formalinized culture Preparations After 3 U tube passages at 25 C bacteria were *cultured over night in BEB at 25 C and 37 C respectively* A platinum loop ful of each broth culture was then sub cultured each loopful in 100 ml of fresh broth The new cultures were maintained at 25 C and 37 C in water baths during continuous shaking For electron microscopy 5 ml samples were taken the cells were washed once with re distilled water and then negatively stained with PTA as described above and used for grid preparations Controls with grid preparations made directly from negatively stained broth cultures were also examined so as to ensure that the flagella were not affected by handling of the cells

The motility of the original over night broth cultures was studied on BEB agar plates at the respective temperatures In some cases cells gently harvested from these plates in sterile re distilled water were examined under the electron micro scope

A few poorly motile strains were investigated repeatedly and after different lengths of incubation in order to find motile cells

Investigation of flagellated cultures after temperature shift from 25 C to 37 C U tube passed cells of the strains Bojsen Møller 40 Becht 51 and MT 79b were used in the tests Each strain was inoculated into 100 ml of BFB previously passed through a 1 0 μ membrane filter (Sartorius Membrane Filter Type SM 11003 34/Gottingen Deutschland) The broth culture was incubated over night at 25 C during continuous shaking in a water bath and to a cell density of about 10^8–10^9/ml (viable counts) Half of the culture was then added to 250 ml of fresh BEB 25 C while the other half was transferred to 250 ml of fresh BEB prewarmed to 37 C. The cultures were then incubated during shaking at the respective temperatures At intervals growth was determined by viable counts and measurements of dry weight The pH during growth was continuously registered throughout the observation period of 6 hr For viable counts 0 1 ml samples were collected serially diluted 0 1 ml volumes were spread on human blood agar plates with glass beads and counted after 48 hr at 37 C *Five fold plate series from each dilution were counted* Dry weigths were determined in 10 ml samples centrifuged at 4 C 2000 g for 45 minutes washed once with re distilled water re centrifuged in the same way re suspended in 0 5 ml of re distilled water and dried for 180 minutes at 80 C

Bacterial flagella were examined in the electron microscope after negative stain ing with PTA For staining 1 ml samples of broth culture were mixed with an equal volume of a 2 per cent PTA solution pH 7 0 The approximate number of flagellated cells *(per cent) and the average number of flagella per cell (total num* ber of cells) were calculated from counts of at least 200 cell from each preparation

Electron microscopy Formvar and carbon coated copper grids prepared in the way described above were examined in an electron microscope Hitachi HS 70 The proportion of flagellated cells and the number of flagella per cell were determined directly during microscopy The wavelengths (WL) the amplitudes (A) and the lengths of *individual flagella were measured from electron micrographs* The formula *given by Leifson et al* (1955) was *used for calculating the spiral* unit lengths (SUL) of the flagella

RESULTS

Flagellation at 25 C

Peritrichous flagella apparently randomly distributed from the bacterial soma could be demonstrated in all of the types of *Yersinia enterocolitica* studied here

Quantitatively however the flagellation differed from one group of strains to another The percentage of flagellated cells in the different strains and maximum number of flagella observed per cell in 48 hr batch cultures at 25 C are given in Table 1 It is clear from the table

during the same time of observation Electron microscopy of cells from poorly motile strains harvested from the semi solid agar plates showed the same low proportion of flagellated cells as did investigation of cells from the broth cultures

Flagellation at 37° C

As shown in Table 1 the strains flagellated when incubated at 25 C had practically no flagellated cells when cultured at 37 C under otherwise identical conditions regarding starting material availability and pH of the medium and duration of culture Single flagellated cells observed in two strains had only one single flagellum per cell

To assess the loss of flagella during culture at 37 C 3 very motile type strains representing different serological and/or biochemical variants were selected and studied regarding their flagellation for a 6 hr period after transfer of 25 C—adapted culture to 37 C The effects of this change in temperature are given in Fig 1 a–c where the decrease in the number of flagella per total number of cells respectively the decrease in the percentage of flagellated cells are given in relation to the average number of generations in the culture calculated from log viable counts/ml It is shown in the figures that the sudden temperature shift influenced the bacterial growth in a somewhat different way in the three experiments The strain Becht 51 gave an average generation number of 2 5 during the observation period of 6 hours During this time the number of flagellated cells successively decreased from 85 per cent to about 19 per cent and the mean number of flagella per cell (of the total number of cells) fell from 1 8 to 0 3 As to strain M X 79b the change in temperature was first followed by a reduction in the number of viable cells by about 50 per cent After a subsequent indefinite lag period the culture yielded on the average 3 1 generations as calculated from the increase of viable counts in the interval between 60 and 360 minutes of the observation period The number of flagellated cells decreased during the same period from about 78 to 19 per cent and the mean number of flagella per cell from 1 6–0 2 Strain Bojsen Moller 70 finally showed a very flat growth curve with on the average only 1 4 generations within 360 minutes The number of flagellated cells decreased during this time from 91 to 66 per cent with a simultaneous decrease of the mean number of flagella per cell from 3 1–1 0 Controls of cultures kept at 25 C but under otherwise identical conditions as those cultured at 37 C invariably showed more than 90 per cent flagellated cells after 6 hours and if anything an increase in the mean number of flagella per cell

TABLE 2

Mean Wavelength (WL), Amplitude (A) and Spiral Unit Length (SUL) of Flagella of Selected Strains of Various Groups of Yersinia enterocolitica *Data in μ Calculated from Electron Micrographs of Formalinized Cells from Tryptose Glucose Broth Cultures 48 hr 25 C Negatively Stained with Potassium Phosphotungstate*

Strain	WL	SD	A	SD	SUL	SD	N
Lucas 404	3.08	–	0.28	–	3.20	–	3
Dickinson 07	2.79	–	0.20	–	2.85	–	2
MY 0	1.24	–	0.14	–	1.47	–	3
MY 134	1.24	–	0.14	–	1.31	–	2
Becht 51	2.83	–	0.31	–	2.99	–	3
Daniels 924	2.78	–	0.27	–	2.90	–	3
B Knox 1017/60–61	2.77	–	0.23	–	2.87	–	2
MY 79b	2.88	0.63	0.33	0.10	3.07	0.69	10
Group mean	2.82	0.05§	0.29	0.04§	2.95	0.03§	
Ye 123 Vache	2.64	0.35	0.25	0.17	2.76	0.42	5
Bojsen Møller 70	2.80	0.17	0.28	0.06	2.94	0.21	10
Borg Petersen 6613	2.83	0.41	0.27	0.08	2.96	0.45	8
Albany 33114	3.05	–	0.31	–	3.20	–	2
Albany 5819	2.80	0.08	0.31	0.04	2.97	0.04	5
Group mean	2.82	0.15§	0.28	0.03§	2.97	0.11§	

§ SD of strain means from group mean
N = number of measurements

TABLE 3

Wavelengths (WL), Amplitudes (A) and Spiral Unit Lengths (SUL) of Flagella in 1 Strain of Yersinia enterocolitica *Data in μ Obtained from Electron Micrographs of Formalin Fixed and Non Fixed Cells from 48 hr Broth Cultures 25 C*

Strain	Preparation		WL	A	SUL
Borg Petersen SP 6613	Formalinized culture		2.96	0.41	3.23
			2.82	0.30	2.97
			2.82	0.26	2.94
			1.94	0.12	1.98
			2.78	0.28	2.92
			2.96	0.26	3.07
			2.90	0.26	3.07
			3.38	0.30	3.51
		Mean	2.83	0.27	2.96
		SD	0.41	0.08	0.4
Borg Petersen SP 6613	Non formalinized culture		3.07	0.32	3.23
			2.71	0.24	2.81
			2.97	0.33	3.14
			2.86	0.26	2.97
			3.02	0.34	3.20
			2.50	0.22	2.59
			2.81	0.21	2.89
		Mean	2.85	0.27	2.98
		SD	0.20	0.05	0.25

that the strains Daniels 1028 and Lucas 404 (isolated from hare) strain Dickinson 07 (from pig) strains MY 0 2 57 60 134 (from man) and strain Becht Hund (from dog) had few or no flagellated cells In the case of these strains examination of the cultures after shorter or longer incubation varying between 6–144 hours revealed no increase in the percentage of flagellated cells One strain (MY 57) gave however about 4 per cent flagellated cells in one of several repeated attempts to segregate motile clones

TABLE 1

Flagellation in Yersinia enterocolitica Strains of Various Types Calculated from Electron Microscopic Counts on Negatively Stained Cells from 48 hr broth cultures at 25 C and 37 C At least 500 Cells of each Strain were Observed

Strain	Biotype	Proportion of flagellated cells (%) 25 C	Proportion of flagellated cells (%) 37 C	Observed maximum number of flagella/cell
Daniels 1028	NO_3	0.1	–	1
Lucas 404	NO_3	0.2	–	3
Dickinson 07	ind xyl	0.3	–	3
MY 0	ind xyl	–	–	–
MY 2	ind xyl	0.1	–	1
MY 57	ind xyl	–(4)	–	–(4)
MY 60	ind xyl	–	–	–
MY 134	ind xyl	0.3	–	2
Becht Hund 200	ind xyl		–	–
Becht 51	ind xyl	70	–	≥ 8
Daniels 924	ind xyl	74	–	≥ 18
B Knox 1017/60 61	ind xyl	39	–	6
MY 39	ind xyl	90	0.2	≥ 10
MY 79b	ind xyl	69	0.4	8
Ye 123 Vache	ind xyl	12	–	7
Bojsen Møller 70	ind xyl	80	–	≥ 18
Borg Petersen SP 6613	ind xyl	89	–	≥ 10
Albany 33114	ind xyl	20	–	6

Figures within brackets denote the flagellation in a culture obtained after repeated attempts to segregate motile clones

As regards ind− xyl+ strains isolated from chinchilla ind− xyl+ strains of human origin (antigen type 9) and ind+ strains of mixed origins the number of flagellated cells varied between 12 and 90 per cent Also the number of flagella per flagellated cell differed in that the cells in poorly flagellated strains had only a single flagellum exceptionally 2-4 flagella per cell while the others were not infrequently richly flagellated but mostly with 2–6 flagella per cell

The motility of the cultures judged from their spreading on a semi solid agar surface varied with the degree of flagellation Strains with high or fairly high percentage of flagellated cells gave growth spreading over the entire surface of the plate in 1–3 days at 25 C while strains belonging in the poorly flagellated group showed no swarming

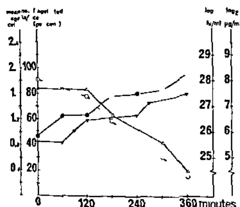


FIG 1a

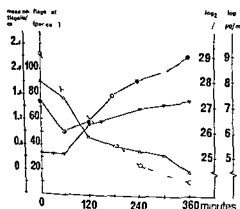


FIG 1b

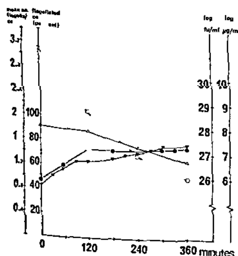


FIG 1c

Fig 1a–c

Decrease in percentage of flagel lated cells and mean number of flagella per cell (total cell num ber) during a 6 hr culture period after temperature shift from 25 C to 37 C in 3 strains of *Yersinia enterocolitica*

●—● log colony forming units (cfu) per ml
▼—▼ log dry weight (µg per ml)
○—○ mean number of flagella per cell (total number of cells)
△—△ percentage of flagellated cells

Fig 1a
Strain Becht 51

Fig 1b
Strain MY 79b

Fig 1c
Strain Bojsen Møller 70

during the same time of observation Electron microscopy of cells from poorly motile strains harvested from the semi solid agar plates showed the same low proportion of flagellated cells as did investigation of cells from the broth cultures

Flagellation at 37 C

As shown in Table 1 the strains flagellated when incubated at 25 C had practically no flagellated cells when cultured at 37 C under otherwise identical conditions regarding starting material availability and pH of the medium and duration of culture Single flagellated cells observed in two strains had only one single flagellum per cell

To assess the loss of flagella during culture at 37 C 3 very motile type strains representing different serological and/or biochemical variants were selected and studied regarding their flagellation for a 6 hr period after transfer of 25 C—adapted culture to 37 C The effects of this change in temperature are given in Fig 1 a–c where the decrease in the number of flagella per total number of cells respectively the decrease in the percentage of flagellated cells are given in relation to the average number of generations in the culture calculated from log viable counts/ml It is shown in the figures that the sudden temperature shift influenced the bacterial growth in a somewhat different way in the three experiments The strain Becht 51 gave an average generation number of 2 5 during the observation period of 6 hours During this time the number of flagellated cells successively decreased from 85 per cent to about 19 per cent and the mean number of flagella per cell (of the total number of cells) fell from 1 8 to 0 3 As to strain M Y 79b the change in temperature was first followed by a reduction in the number of viable cells by about 50 per cent After a subsequent indefinite lag period the culture yielded on the average 3 1 generations as calculated from the increase of viable counts in the interval between 60 and 360 minutes of the observation period The number of flagellated cells decreased during the same period from about 78 to 19 per cent and the mean number of flagella per cell from 1 6–0 2 Strain Bojsen Moller 70 finally showed a very flat growth curve with on the average only 1 4 generations within 360 minutes The number of flagellated cells decreased during this time from 91 to 66 per cent with a simultaneous decrease of the mean number of flagella per cell from 3 1–1 0 Controls of cultures kept at 25 C but under otherwise identical conditions as those cultured at 37 C invariably showed more than 90 per cent flagellated cells after 6 hours and if anything an increase in the mean number of flagella per cell

TABLE 2

Mean Wavelength (WL), Amplitude (A) and Spiral Unit Length (SUL) of Flagella of Selected Strains of Various Groups of Yersinia enterocolitica. Data in μ Calculated from Electron Micrographs of Formalinized Cells from Tryptose Glucose Broth Cultures 48 hr 25 C Negatively Stained with Potassium Phosphotungstate

Strain	WL	SD	A	SD	SUL	SD	N
Lucas 404	3.08	–	0.[illegible]8	–	3.20	–	3
Dickinson 07	2.79	–	0.20	–	2.85	–	2
MY 0	1.24	–	0.14	–	1.47	–	3
MY 131	1.24	–	0.14	–	1.31	–	2
Becht 51	2.83	–	0.31	–	2.99	–	3
Daniels 924	2.78	–	0.27	–	2.90	–	3
B Knox 1017/CO 61	2.77	–	0.23	–	2.87	–	2
MY 79b	2.88	0.63	0.33	0.10	3.07	0.69	10
Group mean	2.82	0.05§	0.29	0.04§	2.9[illegible]	0.09§	
Ye 123 Vache	2.64	0.35	0.2[illegible]	0.17	2.76	0.42	6
Bojsen Møller 70	2.80	0.17	0.28	0.06	2.94	0.21	10
Borg Petersen 6613	2.83	0.41	0.27	0.08	2.96	0.45	8
Albany 33114	3.05	–	0.31	–	3.20	–	2
Albany 5819	2.80	0.08	0.31	0.04	2.97	0.04	5
Group mean	2.82	0.15§	0.28	0.03§	2.97	0.16§	

§ SD of strain means from group mean
N = number of measurements

TABLE 3

Wavelengths (WL), Amplitudes (A) and Spiral Unit Lengths (SUL) of Flagella in 1 Strain of Yersinia enterocolitica. Data in μ Obtained from Electron Micrographs of Formalin Fixed and Non Fixed Cells from 48 hr Broth Cultures 25 C

Strain	Preparation	WL	A	SUL
Borg Petersen SP 6613	Formalinized culture	2.96	0.41	3.23
		2.82	0.30	2.97
		2.82	0.26	2.94
		1.94	0.12	1.98
		2.78	0.28	2.9[illegible]
		2.90	0.26	3.07
		2.90	0.[illegible]	3.07
		3.38	0.30	3.51
	Mean	2.83	0.27	2.90
	SD	0.41	0.08	0.45
Borg Petersen SP 6613	Non Formalinized culture	3.07	0.32	3.23
		2.71	0.24	2.81
		2.97	0.33	3.14
		2.80	0.26	2.97
		3.[illegible]0	0.34	3.[illegible]0
		2.50	0.22	2.59
		2.81	0.21	2.89
	Mean	2.8[illegible]	0.27	2.98
	SD	0.20	0.05	0.2[illegible]

during the same time of observation Electron microscopy of cells from poorly motile strains harvested from the semi solid agar plates showed the same low proportion of flagellated cells as did investigation of cells from the broth cultures

Flagellation at 37 C

As shown in Table 1 the strains flagellated when incubated at 25 C had practically no flagellated cells when cultured at 37 C under otherwise identical conditions regarding starting material availability and pH of the medium and duration of culture Single flagellated cells observed in two strains had only one single flagellum per cell

To assess the loss of flagella during culture at 37 C 3 very motile type strains representing different serological and/or biochemical variants were selected and studied regarding their flagellation for a 6 hr period after transfer of 25 C—adapted culture to 37 C The effects of this change in temperature are given in Fig 1 a–c where the decrease in the number of flagella per total number of cells respectively the decrease in the percentage of flagellated cells are given in relation to the average number of generations in the culture calculated from log viable counts/ml It is shown in the figures that the sudden temperature shift influenced the bacterial growth in a somewhat different way in the three experiments The strain Becht 51 gave an average generation number of 2 5 during the observation period of 6 hours During this time the number of flagellated cells successively decreased from 85 per cent to about 19 per cent and the mean number of flagella per cell (of the total number of cells) fell from 1 8 to 0 3 As to strain MY 79b the change in temperature was first followed by a reduction in the number of viable cells by about 50 per cent After a subsequent indefinite lag period the culture yielded on the average 3 1 generations as calculated from the increase of viable counts in the interval between 60 and 360 minutes of the observation period The number of flagellated cells decreased during the same period from about 78 to 19 per cent and the mean number of flagella per cell from 1 6–0 2 Strain Bojsen Moller 70 finally showed a very flat growth curve with on the average only 1 4 generations within 360 minutes The number of flagellated cells decreased during this time from 91 to 66 per cent with a simultaneous decrease of the mean number of flagella per cell from 3 1–1 0 Controls of cultures kept at 25 C but under otherwise identical conditions as those cultured at 37 C invariably showed more than 90 per cent flagellated cells after 6 hours and if anything an increase in the mean number of flagella per cell

TABLE 2

Mean Wavelength (WL) Amplitude (A) and Spiral Unit Length (SUL) of Flagella of Selected Strains of Various Groups of Yersinia enterocolitica *Data in μ Calculated from Electron Micrographs of Formalinized Cells from Tryptose Glucose Broth Cultures 48 hr 25 C Negatively Stained with Potassium Phosphotungstate*

Strain	WL	SD	A	SD	SUL	SD	N
Lucas 404	3 08	–	0 28	–	3 20	–	3
Dickinson 07	2 79	–	0 20	–	2 85	–	2
MY 0	1 24	–	0 14	–	1 47	–	3
MY 134	1 24	–	0 14	–	1 31	–	2
Becht 51	2 83	–	0 31	–	2 99	–	3
Daniels 924	2 78	–	0 27	–	2 90	–	3
B Knox 1017/60–61	2 77	–	0 23	–	2 87	–	2
MY 79b	2 88	0 63	0 33	0 10	3 07	0 69	10
Group mean	2 82	0 05§	0 29	0 04§	2 9	0 09§	
Ye 123 Vache	2 64	0 35	0 25	0 17	2 76	0 42	5
Pojsen Moller 70	2 80	0 17	0 28	0 06	2 94	0 21	10
Borg Petersen 6613	2 83	0 41	0 27	0 08	2 96	0 45	8
Albany 33114	3 05	–	0 31	–	3 20	–	2
Albany 5319	2 80	0 08	0 31	0 04	2 97	0 04	5
Group mean	2 82	0 15§	0 28	0 03§	2 97	0 16	

§ SD of strain means from group mean
N = number of measurements

TABLE 3

Wavelengths (WL) Amplitudes (A) and Spiral Unit Lengths (SUL) of Flagella in 1 Strain of Yersinia enterocolitica *Data in μ Obtained from Electron Micrographs of Formalin Fixed and Non Fixed Cells from 48 hr Broth Cultures 25 C*

Strain	Preparation	WL	A	SUL
Borg Petersen SP 6613	Formalinized culture	2 90	0 41	3 23
		2 82	0 30	2 97
		2 82	0 26	2 94
		1 94	0 12	1 98
		2 78	0 28	2 92
		2 96	0 26	3 07
		96	0 26	3 07
		3 38	0 30	3 51
	Mean	2 83	0 27	2 96
	SD	*0 41*	*0 08*	*0 45*
Borg Petersen SP 6613	Non formalinized culture	3 07	0 32	3 23
		2 71	0 24	2 81
		2 97	0 33	3 14
		86	0 26	2 97
		3 02	0 34	3 20
		2 50	0 22	2 59
		2 81	0 21	2 89
	Mean	2 85	0 27	2 98
	SD	0 20	0 0	0 25

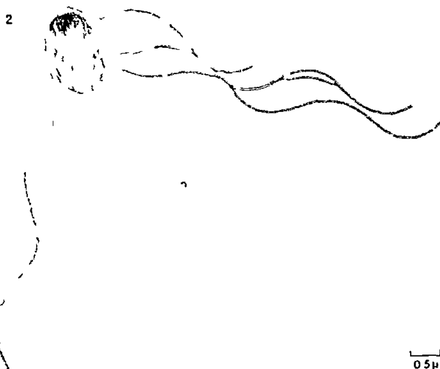


Fig. 2

Strain Bojsen Møller 70 and O antigen type 6, human origin. Flagella demonstrated after 48 hours incubation in tryptose glucose broth at 25° C. Formalin fixation, negative staining with PTA.

Flagellar Morphology

Some data on the morphology of the flagella of the various strains after formalin fixation are given in Table 2, where wavelengths (WL), amplitudes (A) and spiral unit lengths (SUL) (*Leifson et al.* 1955) of different strains have been recorded. As is apparent from the table and also shown in Figs. 2–7, relatively wide variations in the wave lengths and amplitudes were seen not only within different strains but also often in the case of flagella of one and the same bacterial cell (Fig. 4). Flagella with a double curvature (*Leifson* 1960) were also observed (Fig. 6), and in some cases straight flagella were seen (Fig. 3). Maximum flagellar length in the formalinized preparations of the different strains varied between 7 and 11.6 µ.

Formalin fixation did not decrease the variation of the wavelength or of the amplitude in individual strains as compared with the result of examination of a non fixed culture (Table 3).

Among strains investigated after formalin fixation the strains M Y 134 and M Y 0 showed the average shortest mean wavelength (1.24 µ) with a mean amplitude of 0.14 µ; the number of measurements was however very small owing to the sparsity of the flagella. Measure

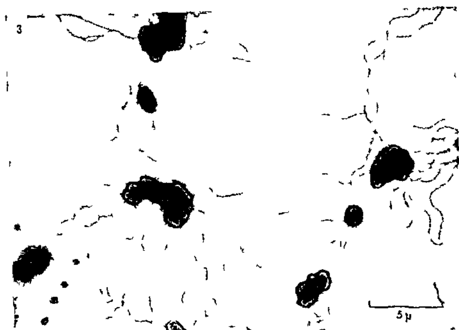


Fig 3

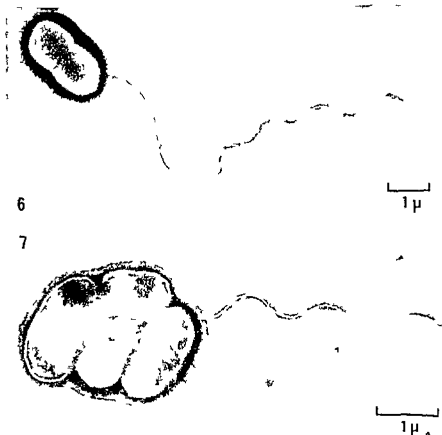
Strain MY 39 ind xyl O antigen type 9 human origin Cells abundantly flagellated and showing several straight flagella Incubation 48 hr in beef extract broth at 25 C Non fixed preparation negative staining with PTA

ments of non fixed preparations of strain MY 57 belonging to the same antigen type 3 gave wavelength means of 1 32 µ (SD 0 14) and 1 24 µ (SD 0 09) after incubation for 48 hours and 96 hours respectively

DISCUSSION

The purpose of the present investigation was twofold *viz* 1) to find out whether divergencies in flagellation or flagellar morphology occur in parallel with differences in motility among strains referred to *Yersinia enterocolitica* and 2) whether such divergencies if any may also be responsible for the variation in the motility of motile strains with temperature

The results did not indicate any differences between various groups of strains with regard to the arrangement of the flagella in agreement with previous observations (*Frederiksen* 1964) peritrichous flagella could be demonstrated in all groups studied Flagellation however was found to differ quantitatively in the different strains The two strains isolated from hare and ind− xyl− strains isolated from man dog and pig showed no flagella or very few flagella compared with the other well flagellated groups Poorly motile human strains as judged from the few possible measurements also showed a shorter wavelength than did strains that were richly flagellated The quanti



Figs 6–7

Fig 6 *Strain* MY 57 and xyl O antigen type 3 human origin Single flagellum with double curvature Incubation for 24 hr in beef extract broth at 25 C Non fixed preparation negative staining with PTA

Fig 7 *Strain* MY 134 and xyl O antigen type 3 human orig n Flagellated cell In formalinized preparation of 48 hr culture in tryptose glucose broth 35 C Negative staining with PTA

tative differences in flagellation were well correlated with the swarming ability of the bacteria on the surface of semi solid agar The results thus suggest that the weak locomotor activity of poorly motile strains is due to lack of flagella rather than to the occurrence of abnormal flagella or to flagellar paralysis

---

Figs 4–5

Strain Daniels 924 and xyl O antigen type 2 chinchilla origin Flagellated cells harvested from semi s lil agar plates 48 hr incubation 25 C showing flagella of different wavelength and amplitude Non fixed preparation negative staining with PTA

A paucity or absence of flagella may be a result of development of non flagellated cells in a certain laboratory environment or of development of non flagellated mutants The influence of different environmental factors such as composition of medium pH age of the culture and temperature on the synthesis and persistance of flagella of different bacteria has long been known (*Ogiuti* 1936 *Weibull & Tiselius* 1945 *Leifson et al* 1955 *Leifson* 1960 *Stocker & Campbell* 1959 *Quadling & Stocker* 1962 *Gerber & Noguchi* 1967) Essential precursors and a suitable source of energy for the synthesis of flagella in a synthetic medium have been studied in *E coli* by *Adler & Templeton* (1967) These authors also demonstrated that the motility was inhibited by heavy metal ions and glucose Also *Leifson* (1960) reported the inhibitory effect of fermentable carbohydrates on the synthesis of flagella

The strains under discussion have been examined in non defined complete media buffered to pH 7 2–7 0 which have invariably given good growth at both 37 C and 25 C It is not likely that there was any deficiency of precursors necessary for the synthesis of flagella It seems more reasonable to assume that medium components present or formed as intermediate metabolites may inhibit the synthesis of flagella of certain biotypes Preliminary experiments have thus shown that EDTA in concentrations between 10^{-4} and 10^{-6} M enhances the motility of strains in the poorly motile group (*Mehn* 1968 unpublished observations) similar observations concerning *E coli* have been reported previously by *Adler & Templeton* (1967)

Poorly flagellated strains examined in cultures of different ages did not show an increased number of flagella in the earlier phases of growth Neither were there any differences between a newly isolated strain and older stock cultures or lyophilized strains

The effect of the temperature on the motility of different *Yersinia enterocolitica* strains has been observed by several investigators (for review see *Jacober* 1968) The present results indicate that the non motility at 37 C of strains motile at 25 C is due to a loss of flagella when cultured at the higher temperature The transfer of cultures from 25 C to 37 C did not result in a complete loss of flagella immediately after the change in temperature but in a successive decrease An exact determination of this decrease as a function of the rate of bacterial growth is possible only if the initial proportion of viable cells of the culture and the number of viable cells per colony forming unit are known The effect of the sudden change in temperature on viability continued cell functions and existing flagella in the different tested cultures is also difficult to assess the initial viable counts after transfer to 37 C differed however only in one case (strain MY 79b) from those of the control culture at 25 C The culture reacted in this case with persisting or transient loss of viability in more than half of the cells But though the results do not allow any detailed analysis of the loss of flagella in relation to cell division they do

suggest a cessation of flagellar synthesis after transfer to the culture to 37 C while synthesis of flagella continued in the control cultures kept at 25 C

Variations in the metabolic activity with temperature have been found for this group of bacteria e g regarding catabolism of certain carbohydrates (*Nilèhn* 1967 a b) or requirements of different growth factors (*Burrows & Gillett* 1966) The mechanism of the possible blocking of synthesis of flagella at the higher temperature is how ever obscure and has as far as is known not been studied

The genetic stability of flagella encountered in single cells in the poorly flagellated group was not studied In one case after repeated attempts to find motile clones one culture yielded about 4 per cent flagellated cells With the aid of micromethods it might be possible under the experimental conditions used to isolate cells yielding a prog eny with stable flagellation

The flagellar shape varied widely in individual strains an observa tion also applying to the closely related *Yersinia (Pasteurella) pseudo tuberculosis* (*Leifson* 1960) Variations in the form of biplicity double curvature or straight flagella were observed both in formalin fixed and in non fixed preparations Whether the differences in mean wave length between certain poorly motile strains of human origin and other groups were significant cannot be decided until a larger number of strains have been studied

It is difficult to assess the possible taxonomic significance of the quantitative and qualitative differences in flagellation in different groups of strains until more knowledge has been obtained of the genetic or physiological background In this conjunction it should however be pointed out that poorly flagellated strains isolated from man dog and pig resemble one another also in biochemical properties (*Nilehn* 1967 b) antigenic components (*Winblad* 1967) and phage sensitivity (*Nicolle et al* 1967 *Nilehn & Ericson* 1969) As an entity the *Yersinia enterocolitica* group seems as regards its flagellation to occupy an intermediate position where the flagellated variants resem ble the otherwise closely related *Yersinia pseudotuberculosis* while strains poor in flagella and above all the biochemically peculiar group of hare origin approach the biochemically related non motile *Yersinia (Pasteurella)* pestis

SUMMARY

Electron microscopic investigation of the flagellation of *Yersinia entero colitica* strains representing various serological and/or biochemical variants showed the occurrence of peritrichous flagella at 25 C in all groups examined

On examination of the strains in cultures in non defined complete media they differed quantitatively in their flagellation There was thus one richly flagellated motile group and one very scantily flagellated

poorly motile group the latter comprising strains belonging to certain biotypes isolated from man dog pig or hare

Richly flagellated strains were often characterized by a relatively large variability in wavelength and amplitude and also in the shape of individual flagella or flagella of single cells

A successive loss of flagella of 3 type strains was observed after transfer of 25 C—adapted cultures to 37 C

REFERENCES

1 Adler J & Templeton B The effect of environmental conditions on the motility of *Escherichia coli* J gen Microbiol 46 175–184 1967

2 Ahlermans J I W M & Terpstra J I Pseudotuberculose bij chinchilla s veroorzaakt door en bijzondere species Tijdschr Diergeneesk 88 91 95 1963

3 Becht H Untersuchungen über die Pseudotuberculose beim Chinchilla Deutsch tierärztl Wschr 69 626–627 1962

4 Burrows T W & Gillett W A The nutritional requirements of some Pasteurella species J gen Microbiol 45 333–345 1966

5 Daniels J J H M & Goudswaard C Enkele Stammen van een op *Pasteurella pseudotuberculosis* gelijkend met geïdentificeerd species geïsoleerd bij knaagdieren Tijdschr Diergeneesk 88 96–102 1963

6 Dickinson A B & Mocquot G Studies on the bacterial flora of the alimentary tract of pigs I *Enterobacteriaceae* and other Gram negative bacteria J appl Bact 24 252–284 1961

7 Frederiksen W C A study of some *Yersinia pseudotuberculosis* like bacteria (*Bacterium enterocoliticum* and *Pasteurella X*) Proc XIVth scand Congr Path Microbiol Oslo 103–104 1964

8 Gerber B R & Noguchi H Volume change associated with the C I transformation of flagellin J Mol Biol 26 197–210 1967

9 Hoeniger J Development of flagella by *Proteus mirabilis* J gen Microbiol 40 29–42 1965

10 Jacober I X I infection à *Yersinia enterocolitica* une Zoonose d avenir Thesis Copedith Paris 1968

11 Leifson E Carhart S & Fulton M Morphological characteristics of flagella of *Proteus* and related bacteria J Bact 69 73–82 1955

12 Leifson E Atlas of bacterial flagellation Academic Press New York/London 1960

13 Loghem J J van The classification of the plague bacillus Antonie v Leeuwenhoek 10 15–16 1944

14 Loghem J J van La classification du Bacille pesteux Ann Inst Pasteur 72 97 1946

15 Mollaret H H & Lucas A Sur les particularites biochimiques des souches de *Yersinia enterocolitica* isolees chez les lievres Ann Inst Pasteur 108 121–125 1965

16 Niolle P Mollaret H H Ham n A & Vieu J F Etude lysogénique bactério lo genique et lysotypique de l espèce *Yersinia enterocolitica* Ann Inst Pasteur 112 86–92 1967

17 Nilehn B Studies on *Yersinia enterocolitica* Characterization of 28 strains from human and animal sources Acta path microbiol scand 69 83 91 1967

18 Nilehn B Bacteriologic studies on 80 strains of *Yersinia enterocolitica* of human origin Internat Symp on Pseudotuberculosis Paris 1967 Symp Series Immunobiol Standard Karger [illegible] New York 9 327–336 1968

19 Nilehn B Sjöström B Damgaard K & Kindmark C O *Yersinia enterocolitica* in patients with symptoms of infectious disease Acta path microbiol scand [illegible] 113 1969

20 Nilehn B & [illegible] Studies on *Y*[illegible] *enterocolitica* Bacteriophages liberated fr[illegible] Acta path microbiol scand 75 [illegible]

21 *Nilehn B* Unpublished observations 1968

22 *Oguti K* Untersuchungen über die Geschwindigkeit der Eigenbewegung von Bakterien Jap J exp Med 14 19 28 1936

23 *Quadling C & Stocker B A D* An environmentally induced transition from the flagellated to the non flagellated state in *Salmonella typhimurium* the fate of parental flagella at cell division J gen Microbiol 28 257–270 1962

24 *Schleifstein J D & Coleman M* An unidentified microorganism resembling *B ligneri* and *Pasteurella pseudotuberculosis* and pathogenic for man N Y St J Med 39 1749–1753 1939

25 *Stocker B A D & Campbell J C* The effect of non lethal deflagellation on bacterial motility and observations on flagellar regeneration J gen Microbiol 20 670 685 1959

26 *Thornley M J & Horne R W* Electron microscope observations on the structure of fimbriae with particular reference to Klebsiella strains by the use of the negative staining technique J gen Microbiol 28 51–56 1962

27 *Weibull C & Tiselius A* Note on acid hydrolysis of bacterial flagella Ark Kemi Min Geol 20 1–4 1945

28 *Winblad S* Studies on serological typing of *Yersinia enterocolitica* The XVth Scand Congr Path Microbiol Copenhagen 115–116 1967

29 *Winblad S* Personal communications 1968

Acta path microbiol scand 77 542–554 1969

The Serum and Toxoid Department Statens Seruminstitut Copenhagen Denmark

BIOSYNTHETIC STABILITY OF THE TOXIGENETIC CAPACITY OF *CLOSTRIDIUM TETANI* ON REPEATED TRANSFER IN CULTURE MEDIA

By

K E NIELSEN

Received 28 V 69

It has often been observed during the preparation of tetanus toxin (*Mueller & Miller* (10) *van Hemert* (6) *Scheibel* (18)) that highly toxigenic *Cl tetani* loose their toxin producing ability to varying extent on repeated subculture

It has been reported recently that repeated transfers of a strain of *Cl perfringens* also resulted in a decrease of the toxigenicity (*Soda et al* (19))

Such instability in biosynthesis is also known from other microbiological fields e g in antibiotic production (*Reusser* (17))

Very little is known concerning the mechanism involved and the factors which cause the decrease in or loss of the toxin producing ability

In order to obtain a highly yield of toxin with the *Cl tetani* strain in question it is necessary to utilize a specific medium (*Mueller & Miller* (9 11 12 13) *Fisek et al* (5) *Latham et al* (7))

As is reported in the above mentioned literature and furthermore by *Mueller & Miller* (8) *Mueller et al* (14) and *Feeney et al* (2 3) the strain has rather complex nutritional requirements However it does not demand such specific conditions for growth as for toxin production since it grows well in different kinds of media without producing more than traces of toxin

It has been observed (*Nielsen* (15)) that the above mentioned decrease in toxigenicity is dependent to some degree on the medium used for subculture This dependency is being investigated and some preliminary results are given in the present paper

MATERIAL AND METHODS

Medium I (special peptone meat infusion)

Peptone meat infusion medium with 0 25 per cent w/v of thioglycollic acid and 5 per cent v/v of Fildes peptic blood broth (horse blood) is made according to the following technique

0 5 kg minced beef muscle is mixed with 0 5 litre tap water stored at + 5 C

overnight and then boiled for 10 minutes and strained through cloth Tap water is added to 1 0 litre and the infusion is boiled for 10 minutes 10 g peptone (Orthana Special Peptone) 3 g sodium chloride and 2 g sec sodium phosphate (with 12 H O) are then added This mixture is boiled for 5 minutes and the pH is adjusted to 7 5 with *sodium hydroxide Filtration is made through paper and then* through Seitz filter The volume is adjusted to 1 0 litre with distilled water and the mixture heated to 100 C for 10 minutes The pH should be 7 4 To this infusion is added 0 ?5 per cent w/v thioglycollic acid and 5 per cent w/v Fildes peptic blood broth Fildes peptic blood broth is made from horse blood with some minor modification of the original prescription (*Fildes* (4)) the digestion being carried out at 56 C for six hours only The final medium is filled into test tubes or containers and autoclaved

Medium I is occasionall used as a semi solid product obtained by the addition of 0 ? per cent w/v agar

The Cl tetani strain in question grows rapidly and copiously in this medium but produces only traces of toxin

Medium II (current medium for toxin production)

This is a slightly modified form of the medium described by *Mueller & Miller* (11) A commercial product Tryptone Oxoid i used instead of pancreatic digest of casein or N Z Case and soluble ferrous sulphate is used instead of reduced iron The composition of the medium is shown in Table 1

TABLE 1

Composition of Medium II which is a slight Modification of the Mueller & Miller Medium for Tetanus Toxin Production

Components	*per litre*	
Tryptone Oxoid	25 0 g	
Ox heart extract	50 0 ml	equivalent to 45 8 g of fresh ox heart
Glucose	11 0 g	
NaCl	2 5 g	
Na HPO	2 0 g	
KH PO	0 15 g	
MgSO 7H O	0 15 g	
l cystine	0 25 g	
l tyrosine	0 5 g	
Ca pantothenate	1 0 mg	
Uracil	2 5 mg	
Thiamine	0 25 mg	
Riboflavine	0 5 mg	
Pyridoxine	0 ?5 mg	
Biot n	5 mcg	
FeSO 7H O	40 0 mg	
D stilled water	to 1 0 litre	

This medium in which the *Cl tetani* strain in question both grows well and produces high concentrat on of toxin has been us d for many years in the routine production of tetanus toxin at Staten Se uminstitut Copenhagen

It has been observed that the yield of tox n may vary with the batch of Tryptone Oxoid used Ho e er prelim nary *tr atment of the tryptone with charcoal* eliminated most of th e variat ons The optimum effect of charcoal treatment is obtained by adding 7 g charcoal (Norit) per litre of a 10 per cent Tryptone Oxoid solution in dist lled water

In some experiments medium II with an add tion of 0 25 per cent w/v thioglycollic acid was used in rder to obtain a lower redox potential

Med um III (semi solid g r bou llon with glucose)

Meat infusion broth with 1 pc c nt w/v peptone (Orthana special) 0 3 per c nt

w/v NaCl 0 2 per cent w/v Na_2HPO_4 $12H_2O$ 0 2 per cent w/v glucose and 0 2 per cent w/v agar

Strain

The non sporulating strain of *Cl tetani* used in this work has been used for routine production of tetanus toxin at Statens Seruminstitut Copenhagen since 1951 and also in many other countries

The strain originated from a freely sporulating strain collection No 298 which Dr Howard Mueller Harvard Medical School Boston received from the Division of Laboratories on Research New York State Health Department Albany N Y in 1939

In Dr Mueller s laboratory the strain no longer sporulated freely after daily subculture for some months in peptone meat infusion broth with 1 per cent glucose in the presence of CO_2 and H_2

A culture of the non sporulating variant No 43415 was returned to Albany in 1943 from which source it was received in this laboratory in 1949

In our laboratory the strain is kept as a lyophilized culture made from a two day transfer in medium III

Cultivation Methods

1) *Subculture* The above mentioned media were used for subculture as described under each experiment and cultivation was performed at 34 C in test tubes (155 mm high internal diameter 19 mm) containing 10 ml medium The amount of inoculum was 0 2 ml The cultivation time is stated under each experiment this was usually from 1 to 3 days Each transfer was controlled for purity on blood agar dishes both aerobically and anaerobically

2) *Toxigenicity testing* (toxin production) The ability to produce tetanus toxin was investigated by cultivation in test tubes under the same conditions as those mentioned above except that the growth period was prolonged to 10 days Filtration through ordinary filter paper was then carried out toluene was added to the filtrate and toxin titration was performed Toxigenicity testing was carried out either in medium II (without thioglycollic acid) or in medium I with and without agar

Titration of Toxin in vitro

1) *Flocculation (Lf)* The test was carried out by the Ramon or Lf method *i e* varying amounts of a local reference antitoxin (0 1–1 0 ml with a dose interval of 0 10 log) were mixed with a constant amount (1 0 ml) of the toxin to be tested The tubes were placed in waterbath at 48 C until floculation had taken place The titre is calculated from the first tube showing flocculation and expressed in Lf/ml The limit of error under these conditions is about ± 10 per cent of the observed result

2) *Mixed floculation* The toxin to be tested was mixed in a ratio of 1 1 with a local reference toxin known to give only specific flocculation with the antitoxin used Other conditions were as described under point 1

The mixed flocculation technique prevents to a certain degree *misreading* caused by false flocculation zones In spite of the much wider limit of error especially with low toxin values (for Lf about 3–6 ± 100 per cent for Lf about 10 ± 62 per cent of the observed value) as compared to those of the direct Ramon test this method is useful for estimation of the Lf titre in toxins of low value *i e* below 10 Lf per ml with which it is difficult to obtain flocculation by the direct method

The mixed flocculation technique was used as supplementary test in all cases where there was reason to doubt the specificity of the direct flocculation and where more than one floculation zone occurred Thus the Lf titres given in the curves indicate with reasonable certainty the specific antitoxin combining capacity in the various filtrates In most of the experiments this has been verified by in vivo titrations

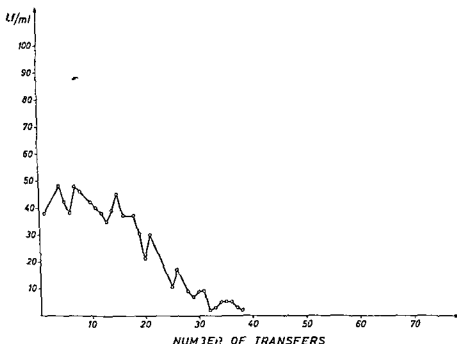


Fig 1

Decrease in toxigenicity by subculture of Cl tetani strain 43415 in medium II using 38 transfers over a period of 76 days
Open circles represent the Lf per ml in a 10 day old culture in medium II of the corresponding transfer
Abscissa Number of transfers
Ordinate Lf/ml

Titration of toxin in vivo

Antitoxin neutralization (L) The test was carried out in white mice (weight 16–18 g) by injecting mixture of one tenth of an international antitoxin unit and varying amounts of the toxin to be tested the endpoint of the titration being the mixture that killed 50 per cent of the animals in five days

Generally dose intervals of 0 10 log were used and two mice were injected per dose Statistical evaluation of a series of titrations in our laboratory has disclosed that the limit of error under these conditions was ± 1? per cent of the observed value

The L titre of tetanus toxin is generally expressed in L /10/50 per ml being assayed against one tenth of an antitoxin unit However to facilitate comparison with the Lf titre which is based on a level of one antitoxin unit the number of L /10/50 per ml found by the in vivo titration is divided by 10 and shown as L /1/50 in the present work

RESULTS

Successive subculture of *Cl tetani* strain 13415 in medium II led to a systematic loss in toxigenicity Fig 1 shows the result of such an

Thanks are due to *Mr Weis Bentzon* Biostatistical Department for making the statistical evaluations

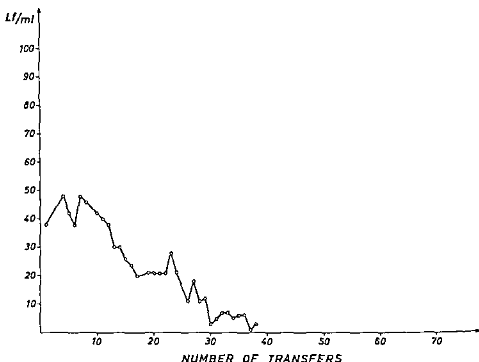


Fig 2

Decrease in toxigenicity by subculture of Cl tetani strain 43415 in medium II containing 0 25 per cent w/v thioglycollic acid Open circles represent the Lf per ml in a 10 day old culture of the corresponding transfer The first transfers are the same as in Fig 1 From transfer No 13 thioglycollic acid was added to the medium The 38 transfers cover a period of 76 days
Abscissa Number of transfers
Ordinate Lf/ml

experiment where 38 transfers were made over a period of 76 days (3 times weekly)

The toxigenicity of the subculture was tested in medium II as described under toxigenicity testing The open circles represent the toxin production of the strain expressed in Lf/ml It will be seen that after about 15 transfers a marked decrease in Lf occurred and that after about 30 transfers only very low values were obtained

The Lf titres at the beginning and end of this and the next experiment were checked by in vivo titration and the two parameters showed a reasonable degree of agreement

Fig 2 shows the results of a similar experiment except that after transfer No 13 0 25 w/v per cent thioglycollic acid was added to the medium The resulting lower redox condition did not seem to influence essentially the loss in toxigenicity during the subsequent subcultures

As the decrease in toxigenicity progressed unspecific flocculation zones occurred with increasing frequency

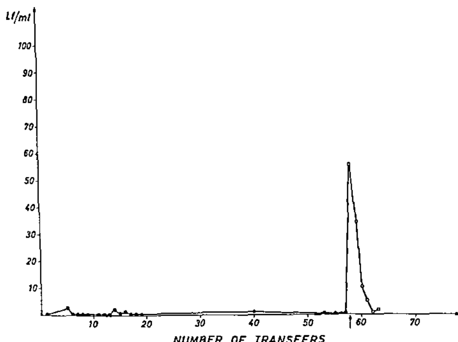


Fig 3

Influence on the toxigenicity of Cl tetani strain 43415 of 57 transfers in medium I with agar and 5 additional transfers in medium II
The dots show the toxin production in Lf per ml in a 10 day old culture in medium I of the corresponding transfer of the Cl tetani strain 43415 in medium I with agar
The open circles indicate the toxin production in a 10 day old culture in medium II of the corresponding subculture
Abscissa Number of transfers
Ordinate Lf/ml

As reported previously (15) the decrease in toxigenicity of *Cl tetani* strain 43415 by repeated subculture in semi solid agar bouillon with glucose (medium III) was much less marked than when subcultured in medium II

When subculture was carried out in medium I in which as mentioned the strain grows exceptionally fast and gives copious growth but produces only minute amounts of toxin the toxigenicity appeared to be stable through a considerable number of transfers

In the present work the number of consecutive subcultures in medium I was extended to more than 100 over a period of more than 30 weeks without any demonstrable decrease in toxigenicity as judged by 10 day old cultures in medium II

However some change in the metabolic properties of the strain seems to have taken place since after a number of transfers in medium I the toxigenicity decreased at an accelerated rate on subsequent subculture in medium II A typical experiment to demonstrate this is

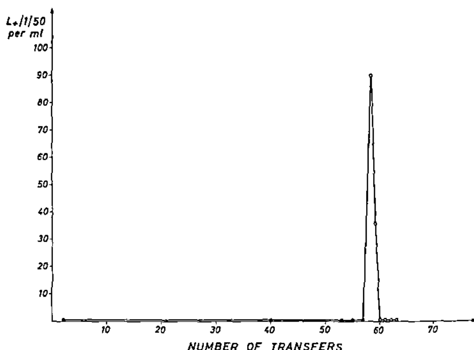


Fig 4

In vivo titration of the same culture filtrates as shown in Fig 3 The toxin production is given in L /1/50 units per ml to enable a comparison with the Lf titres in Fig 3
Abscissa Number of transfers
Ordinate L /1/50 per ml

shown in Fig 3 The strain was transferred 57 times in medium I with 0 2 per cent of agar the transfers being carried out every second day over a period of 17 weeks

The dots in the diagram show the toxin production in Lf per ml obtained after 10 days growth of the corresponding transfers in medium I without agar As can be seen the strain produced only very little toxin in this medium over the whole period

After transfer No 57 the strain was transferred to medium II in which five additional transfers were performed The toxin production in Lf per ml in a 10 day old culture in medium II of the corresponding transfers is indicated by the open circles

Transfer No 57 gave a high yield of toxin in medium II but after only one subculture in this medium there was a decrease in toxin production from 56 to 34 Lf/ml After three to four transfers in medium II the toxin production had decreased to a very low level As can be seen from Figs 1 and 2 it took more than 25 transfers to invalidate the toxigenicity to the same degree without previous subculture in medium I

In this experiment in vivo titrations of each batch were also carried

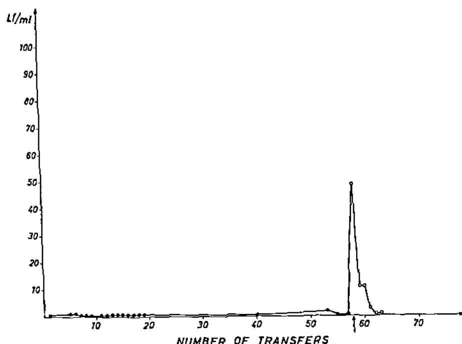


Fig 5

Influence on the toxigenicity of Cl tetani 43415 of 57 transfers in medium I without agar on additional transfers in medium II
The dots show the toxin production in medium I without agar and the open circles the toxin production in medium II of the corresponding subculture
Abscissa Number of transfers
Ordinate Lf/ml

out the results are shown in Fig 4 During the first 10 days culture in medium II the L + titre was significantly higher than the Lf titre whereas that was not the case for the later cultures in this medium However on the whole the antitoxin combining capacity as measured in vivo confirms the result found by Lf titration

Figs 5 and 6 show the results of a similar experiment in which the first 57 transfers were made in medium I without the addition of agar Both the in vivo and the in vivo titration gave results similar to those found in the experiment presented in Figs 3 and 4 Again the L+ titre of the first 10 day old culture in medium II was considerably higher than the Lf titre

It was then investigated whether the low toxigenicity induced by repeated transfers in medium II could be regenerated by subculture in a medium which stabilizes the toxigenicity

For this purpose a medium II culture which was reduced from a toxin producing ability of more than 50 Lf/ml to practically no toxigenicity viz No 46 of a series of successive transfers was subcultured

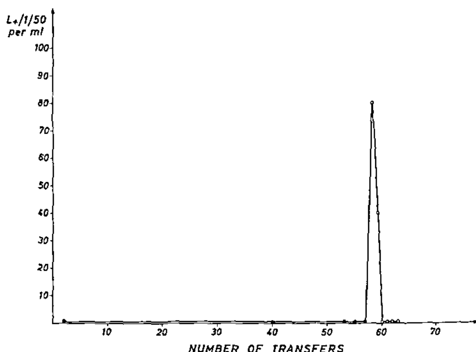


Fig 6

In vivo titration of the same culture filtrates as shown in Fig 5 The toxin production is given in L+/1/50 units per ml to enable comparison with the Lf titres in Fig 5
Abscissa Number of transfers
Ordinate L+/1/50 per ml

three times in medium I with and without addition of agar and in medium III

The third transfer in each medium was then tested for toxin production in medium II in the ordinary way

The results are presented diagrammatically in Fig 7 Only the L+/1/50 titres are given the Lf values as mentioned being difficult to measure with a satisfactory degree of certainty at these low levels

The results for medium I with agar medium I without agar and medium III are shown in Curves A B and C respectively

It was ascertained by toxigenicity testing of the three transfers in each of the three different media that no toxin production which could be measured by the method used took place in any of the transfers On cultivation of the third transfer in medium II some but only very little toxin was produced in comparison with that obtained when the strain was subcultured only in medium I (cf Figs 3 4 5 and 6) The same applied to all three media tested

On further subculture in medium II the acquired slight toxigenicity was again lost after only one transfer

In the next experiment a culture of the lyophilized stock of *Cl tetani*

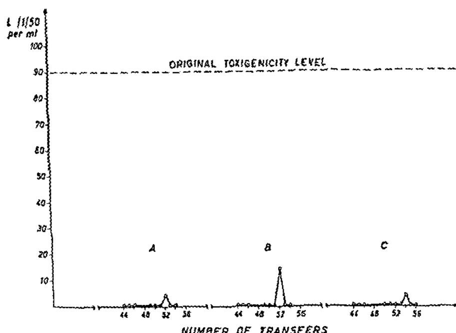


Fig 7

Influence of three successive transfers in medium I with agar medium I without agar and medium III on the toxigenicity of Cl tetani strain 43415 which has lost its toxin producing ability during 46 transfers in medium II
Toxigenicity determined by renewed subculture in medium II
Open circles show the toxin production in medium II dots the toxin production in medium I with agar closed triangles the toxin production in medium I without agar and open triangles the toxin production in medium III all of the corresponding transfers number
Abscissa Number of transfers
Ordinate L+/1/50 per ml

No 43415 used for routine production which had been transferred nine times partly in medium II and partly in medium III and thereby lost about 70 per cent of its original toxigenicity was transferred to medium I with agar and subcultured in this medium during a period of about two years

219 subcultures in medium I (total number of transfers 228) were made at varying intervals and the toxin production in medium II was investigated on a number of these the aim being to ascertain whether prolonged series of transfers in medium I would reestablish an only partly lost toxigenicity

The results is presented in Fig 8 the L+/1/50 titre being the parameter for the toxin production

The titre of the original 10 day-old lyophilized culture was 50 L+/1/50 per ml and that of the ninth subculture in medium II and medium III 15 None of the first 46 transfers in medium I tested for

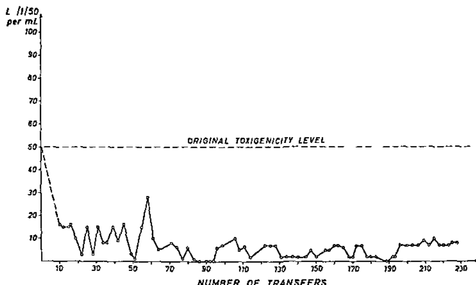


Fig 8

Toxigenicity during 228 transfers of Cl tetani strain 43415 carried out over a period of two years The first nine transfers were made in medium II and medium III by which procedure the strain lost about 70 per cent of its toxigenicity The following 219 transfers were carried out in medium I with agar The toxigenicity indicated by open circles was tested by inoculation in medium II and growth for 10 days
Abscissa Number of transfers
Ordinate L +/1/50 per ml

toxigenicity in medium II exceed that latter level but showed fluctuations around a mean of 10 L+/1/50

Transfer No 49 in medium I (total number of transfers 58) caused an increase in titre to about 30 L+/1/50 per ml after which the titres varied between 0 and about 10 for the rest of the period On the whole there was no indication of a regeneration of the original toxin producing capacity

DISCUSSION

The highly toxigenic strain No 43415 used in the present investigation is probably an asporogenic mutant of the commonly found low toxigenic strains To the writer s knowledge this strain and variants of it are the only highly toxigenic tetanus strain reported hitherto The type of genetic change causing the high toxigenicity is not known It is tempting to assume a bacteriophage as the influencing agent but up to now no report has been made of any bacteriophage connection with the toxigenicity of *Cl tetani* strains One work viz *Cowles* (1) reports a bacteriophage which is active against *Cl tetani*, but this is said to have no influence on the toxigenicity Recently *Prescott & Altenbern* (16) attempted unsuccessfully to isolate bacteriophages from *Cl tetani* after induced lysis

It is thus still an open question whether a highly toxigenic gen residing in a temperate phage or some other transformation system are involved

The results reported in the present paper demonstrate that the highly toxigenic state of *Cl tetani* strain No 43415 is a labile phase to a certain degree and that maintenance of the high toxigenicity on subculture is dependent on the composition of the medium and the number of transfers

The stability of the toxigenicity is best in medium I in which medium the strain grows fast and copiously It is possible to make more than 50 transfers over a period of 3 to 5 months in that medium without any loss in toxigenicity whereas if subculture is carried out in medium II the toxigenicity of the strain will decrease to a very low level after about 30 transfers It has not been possible to regenerate the toxigenicity by the number of transfers in medium I carried out in this work

It should be pointed out that there must be an important difference in the metabolism during growth in these two media since the strain does not produce any or only very small amounts of toxin in medium I but an ample amount in medium II

Furthermore during consecutive transfers in medium I some metabolic change takes place in the cells since the toxigenicity decreases more rapidly by subsequent transfers in medium II About 30 transfers in medium II are needed to cause a gradual decrease to a very low toxigenicity whereas after a number of preceding transfers in medium I this decrease takes place after only two to three transfers

The experiments reported here also demonstrate that the selection of the low toxigenic mutant (or mutants) must be influenced by factors in the medium Selection may be caused either by a biochemical mutation such as a change in the enzymatic systems or by influencing the cell wall receptor mechanism

The improved anaerobic conditions caused by agar and thioglycollic acid did not seem to influence the decrease in toxigenicity

It is to be hoped that some of these problems together with other problems in this field may be elucidated in a better and less time consuming way by continued cultivation experiments Such experiments are in progress at present and the results will be published later

SUMMARY

1 The maintenance of the high toxigenicity of *Clostridium tetani* strain No 4341o in subcultures is dependent on the composition of the medium

2 The toxigenicity of the strain can be maintained for more than 50 transfers over a period of five months in a special peptone meat infusion medium with thioglycollic acid

3 Subculture of the strain in the *Mueller & Miller* medium used for routine production of tetanus toxin reduces its toxigenicity to a very low level after about 30 transfers

4 It was not possible by more than 200 consecutive subcultures in special peptone meat infusion medium with thioglycollic acid carried out over a period of two years to regenerate the toxigenicity of a culture which had lost about two thirds of its toxigenicity by subculture in *Mueller & Miller medium*

5 After consecutive transfers of the strain in special peptone meat infusion medium with thioglycollic acid the decrease in toxigenicity by subsequent transfer in *Mueller & Miller* medium occurred more rapidly

REFERENCES

1 *Cowles P B* A bacteriophage for *Cl tetani* J Bacteriol 27 163–164 1934

2 *Feeney R E Mueller J H & Miller P A* Growth requirements of *Clostridium tetani* II Factors exhausted by growth of the organism J Bacteriol 46 559 562 1943

3 *Feeney R E Mueller J H & Miller P A* Growth requirements of *Clostridium tetani* III A synthetic medium J Bacteriol 46 563–571 1943

4 *Fildes P* A new medium for the growth of *B influenzae* Brit J Exper Path 1 129–130 1920

5 *Fisel A H Mueller J H & Miller P A* Muscle extractives in the production of tetanus toxin J Bacteriol 67 329–334 1954

6 *Hemert P van* Personal communication 1963

7 *Latham W C Bent D F & Levine L* Tetanus toxin production in the absence of protein Appl Microbiol 10 146–152 1962

8 *Mueller J H & Miller P A* Growth requirements of *Clostridium tetani* J Bacteriol 43 763–772 1942

9 *Mueller J H & Miller P A* Production of tetanal toxin J Immunol 50 377–384 1945

10 *Mueller J H & Miller P A* Factors influencing the production of tetanal toxin J Immunol 56 143–147 1947

11 *Mueller J H & Miller P A* Variable factors influencing the production of tetanus toxin J Bacteriol 67 271–277 1954

12 *Mueller J H & Miller P A* Separation from tryptic digests of casein of some acid labile formation J Bacteriol 69 634–642 1955

13 *Mueller J H & Miller P A* Essential role of histidine peptides in tetanus toxin production J Biol Chem 223 185–194 1956

14 *Mueller J H Schoenbach E B Jezukawicz J J & Miller P A* Production of tetanus toxin on peptone free media J Clin Invest 22 315–318 1943

15 *Nielsen K E* Growth and toxin production by *Clostridium tetani* Symp Ser Immunobiol Standard (Karger Basel/New York) 3 207–216 1966

16 *Prescott L M & Altenbern R A* Inducible lysis in *Clostridium tetani* J Bacteriol 93 1220–1226 1967

17 *Reusser F* Stability and degeneration of microbial cultures on repeated transfer Appl Microbiol 5 189–209 Academic Press 1963

18 *Scheibel I* Unpublished results

19 *Soda S Yamato A Ito A & Murata R* Note on the maintenance of toxigenicity of seed cultures of *Clostridium perfringens* PB6K with special reference to alpha toxin Jap J Med Sci Biol 21 91–94 1968

Acta path microbiol scand 77 555-556 1969

BRIEF REPORTS

MILLIPORE® FILTER AS SUPPORT FOR FRESH FROZEN SECTIONS OF PERORAL BIOPSY SPECIMENS OF SMALL INTESTINE

By *Clas G Lindström*

If peroral biopsy specimens of small intestine are to be fixed (*e g* in Bouin s fluid) sectioned and stained the specimen may be placed directly on a firm support such as a fine mesh plastic net from which it can be removed before it is sectioned But if frozen sections of unfixed biopsies are to be prepared for histochemical staining for enzymes for example it is not possible to use such a net or the like An ideal material should provide sufficient support for the specimen and it should be possible to cut it together with the specimen if the latter is to be sectioned parallel to the villi and perpendicular to the luminal surface So far however any suitable material fulfilling these requirements is apparently not known

Filter paper is not suitable because the cellulose fibres split when the paper is cut on the microtom with the result that the histological preparations will be uneven Agar and gelatine have also proved unsuitable because they are hard and difficult to cut when frozen besides which the sections are uneven and liable to split

It was therefore decided to try MF Millipore® filter with a pore size of 5 µ With this material as a support and the use of a suitable embedding medium for cryostat operation *e g* OCT® (Ames LAB TEK) during the actual freezing of the specimen

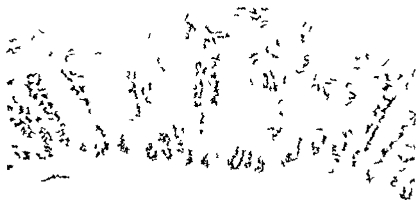


Fig 1

Biopsy from a 3 year old boy with coeliac disease treated with gluten free food Mounted on Millipore filter Fresh frozen section Succinic dehydrogenase X 75 Note the good orientation of the crypts of Lieberkuhn

Received 6 x 69 *from the University Department of Pathology University of Lund General Hospital 214 01 Malmö Sweden*

3 Subculture of the strain in the *Mueller & Miller* medium used for routine production of tetanus toxin reduces its toxigenicity to a very low level after about 30 transfers

4 It was not possible by more than 200 consecutive subcultures in special peptone meat infusion medium with thioglycollic acid carried out over a period of two years to regenerate the toxigenicity of a culture which had lost about two thirds of its toxigenicity by subculture in *Mueller & Miller medium*

5 After consecutive transfers of the strain in special peptone meat infusion medium with thioglycollic acid the decrease in toxigenicity by subsequent transfer in *Mueller & Miller* medium occurred more rapidly

REFERENCES

1 *Cowles P B* A bacteriophage for *Cl tetani* J Bacteriol 27 163–164 1934

2 *Feeney R E Mueller J H & Miller P A* Growth requirements of *Clostridium tetani* II Factors exhausted by growth of the organism J Bacteriol 46 559–562 1943

3 *Feeney R E Mueller J H & Miller P A* Growth requirements of *Clostridium tetani* III A synthetic medium J Bacteriol 46 563–571 1943

4 *Fildes P* A new medium for the growth of *B influenzae* Brit J Exper Path. 1 129–130 1920

5 *Fisel N H Mueller J H & Miller P A* Muscle extractives in the production of tetanus toxin J Bacteriol 67 329–334 1954

6 *Hemert P van* Personal communication 1963

7 *Latham W C Bent D F & Levine L* Tetanus toxin production in the absence of protein Appl Microbiol 10 146–152 1962

8 *Mueller J H & Miller P A* Growth requirements of *Clostridium tetani* J Bacteriol 43 763–772 1942

9 *Mueller J H & Miller P A* Production of tetanal toxin J Immunol 50 377–384 1945

10 *Mueller J H & Miller P A* Factors influencing the production of tetanal toxin J Immunol 56 143–147 1947

11 *Mueller J H & Miller P A* Variable factors influencing the production of tetanus toxin J Bacteriol 67 271–277 1954

12 *Mueller J H & Miller P A* Separation from tryptic digests of casein of some acid labile formation J Bacteriol 69 634–642 1955

13 *Mueller J H & Miller P A* Essential role of histidine peptides in tetanus toxin production J Biol Chem 223 185–194 1956

14 *Mueller J H* Schoenbach F B Jezukawicz J J & Miller P A Production of tetanus toxin on peptone free media J Clin Invest 22 315–318 1943

15 *Nielsen K E* Growth and toxin production by *Clostridium tetani* Symp Ser Immunobiol Standard (Karger Basel/New York) 3 207–216 1966

16 *Prescott L M & Altenbern R A* Inducible lysis in *Clostridium tetani* J Bacteriol 93 1220–1226 1967

17 *Reusser F* Stability and degeneration of microbial cultures on repeated transfer Adv Appl Microbiol 5 189–209 Academic Press 1963

18 *Scheibel I* Unpublished results

19 *Soda S Yamato A Ito A & Murata R* Note on the maintenance of toxigenicity of seed cultures of *Clostridium perfringens* PB6K with special reference to alpha toxin Jap J Med Sci Biol 21 91–94 1968

Acta path microbiol scand 77 555–556 1969

BRIEF REPORTS

MILLIPORE® FILTER AS SUPPORT FOR FRESH FROZEN SECTIONS OF PERORAL BIOPSY SPECIMENS OF SMALL INTESTINE

By *Clas G Lindstrom*

If peroral biopsy specimens of small intestine are to be fixed (*e g* in Bouin s fluid) sectioned and stained the specimen may be placed directly on a firm support such as a fine mesh plastic net from which it can be removed before it is sectioned. But if frozen sections of unfixed biopsies are to be prepared for histochemical staining for enzymes for example it is not possible to use such a net or the like. An ideal material should provide sufficient support for the specimen and it should be possible to cut it together with the specimen if the latter is to be sectioned parallel to the villi and perpendicular to the luminal surface. So far however any suitable material fulfilling these requirements is apparently not known.

Filter paper is not suitable because the cellulose fibres split when the paper is cut on the microtome with the result that the histological preparations will be uneven. Agar and gelatine have also proved unsuitable because they are hard and difficult to cut when frozen besides which the sections are uneven and liable t[illegible] split.

It was therefore decided to try MF Millipore® filter with a pore size [illegible] this material as a support and the use of a suitable embedding medium for cry[illegible] operation *e g* OCT® (Ames LAB TEK) during the actual freezing of the specim[illegible]

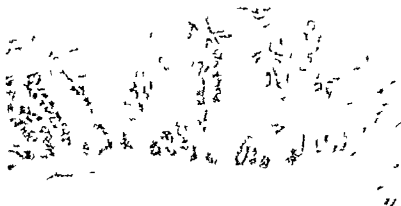


Fig 1

Biopsy from a 3 year old boy with coeliac disease trea[illegible]
Mounted on Millipore filter Fresh frozen section Su[illegible]
Note the good orientation of the crypts of [illegible]

Received 6 x 69 *from the University Department* [illegible]
General Hospital 214 01 Malmo Sweden

Results and Discussion

Duodenum is rapidly labelled by leaking locally injected ^{3}H thymidine because of the rapid proliferation rate of the intestinal epithelial cells. This labelling exceeds greatly the amounts of tritiated DNA brought there by labelled lymphoid cells. During the 48 hour period from the labelling to the death of the animals extensive re-utilization of tritiated DNA does not occur. Therefore the ratio between spec. act. of the locally labelled organ and spec. act. of duodenum is an expression of the efficiency of the local labelling. These ratios were compared with the corresponding ratios in the intravenously labelled animals by Student's t test (Table 1). Good local labelling was obtained both for thymus and bursa in all age groups of animals. Autoradiography of the locally labelled organs showed high frequency of heavily labelled lymphoid cells, mostly small lymphocytes. Some heavily labelled large and medium sized lymphocytes could also be found. In the thymus the heavily labelled cells were most frequent in the cortex and in the bursa the heavily labelled cells were rather uniformly distributed in the follicles.

This method can be used for tracing of thymus and bursa derived cells in the other (lymphoid) organs of the normal chicken. Results of such studies have been reported in part (*Linna et al.* 1968, 1969) and will be reported more extensively (*Hemmingsson & Linna* to be published). The method can also be used to study cell emigration from central lymphoid organs in chickens with stimulation or defects of the immune system.

TABLE 1

Efficiency of Local Labelling of Bursa and Thymus with ^{3}H Thymidine Compared with Incorporation of Intravenously Administered Isotope into these Organs

Ratio	Way of labelling	Age of animals at labelling: 24 hours	6 weeks	3 ½ months
spec. act. bursa / spec. act. duod.	intrabursal	12.0 ± 2.0	16.0 ± 2.0	11.0 ± 2.7
	intravenous	0.35 ± 0.02	0.42 ± 0.03	0.50 ± 0.06
		$p < 0.001$	$p < 0.001$	$p < 0.005$
spec. act. thymus / spec. act. duod.	intrathymic	4.0 ± 0.4	11.0 ± 2.0	10.0 ± 3.3
	intravenous	0.19 ± 0.02	0.14 ± 0.01	0.19 ± 0.01
		$p < 0.001$	$p < 0.001$	$p < 0.005$

Mean ± standard error of the mean

References: *Cooper M D, Peterson R D A, South M A & Good R A*: J Exp Med *123*, 75–102, 1966. — *Linna T J*: Blood *31*, 727–746, 1968. — *Linna T J, Brenning T & Hemmingsson F J*: Exp Hematol *17*, 14, 1968. — *Linna T J, Brenning T & Hemmingsson F J*: *In* Advances of Experimental Medicine and Biology. Plenum Publ Corp, New York, in press. — *Schneider W C*: J Biol Chem *161*, 293–303, 1945. — *Warner N L, Szenberg A & Burnet F M*: Austral J Exp Biol Sci *40*, 373–387, 1962. — *Woods R & Linna T J*: Acta path microbiol scandinav *64*, 470–476, 1965.

Acta path microbiol scand 77 559–560 1969

ISOLATION OF CITRININ AND OXALIC ACID FROM *PENICILLIUM VIRIDICATUM* WESTLING AND THEIR NEPHROTOXICITY IN RATS AND PIGS

By *P Friis E Hasselager* and *P Krogh*

Nephrotoxic compounds were isolated from a strain of *Penicillium viridicatum* Westling This strain was isolated from a batch of barley which by feeding caused chronic kidney degeneration in pigs and rats (*Krogh & Hasselager* 1968) Liquid corn steep medium was inoculated and incubated as still culture for 30 days at 25 C followed by cold incubation at 5 C for 14 days After concentration of the liquid medium fractionation and isolation of nephrotoxic compounds was carried out using white Wistar rats as test animals Water suspensions of the fractions and compounds were administered perorally to rats over a period of 2–4 weeks

Two compounds were found nephrotoxic Citrinin and oxalic acid The citrinin induced kidney damage is characterized by enlarged kidneys histologically hydropic degeneration loss of brush border and pyknotic nuclei are observed in the proximal tubules These lesions are accompanied by thickening of the tubular basement membranes activation of interstitial cells and formation of collagen Some tubules are cystic dilated

This kidney degeneration is comparable to the kidney damage in rats and pigs caused by feeding barley inoculated with the strain of *P viridicatum* as well as by the batch of barley from which the fungus was isolated

In rats long term administration *per os* of oxalic acid and oxalates causes a chronic kidney damage characterized by dilated tubules formation of cysts and connective tissue and scattered crystals of calcium oxalate in the tubules No nuclear alterations were observed

The effect of the 2 nephrotoxic compounds perorally administered to pigs was also studied

EXPERIMENT 1

Administration of Sodium Oxalate

Two pigs of 40 kg body weight received perorally administered sodium oxalate daily 200 and 1000 mg/kg body weight respectively for 42 days The oxalate was mixed with the feed which was readily eaten by the pigs No influence on the growth rate behaviour water consumption serum creatinine and blood urea nitrogen was observed By necropsy of the pigs and subsequent histological examination of all organs only a slight interstitial fibrosis was observed in the kidneys of the pig administered daily 1000 mg sodium oxalate/kg body weight Thus sodium oxalate seems to possess a very low toxicity to pigs

EXPERIMENT 2

Administration of Citrinin

Six pigs of 30 kg body weight received perorally administered citrinin which was mixed with the feed covered by an agar film The administration of citrinin was carried out daily according to the following schedule Two pigs received 20 mg/kg body weight during 34 and 70 days respectively two pigs received 40 mg/kg body weight during 8 and 42 days respectively and two pigs received 100 mg/kg body

Received 8 xi 69 *from the Department of Organic Chemistry Department of Pharmacology and Toxicology Institute of Hygiene and Microbiology the Royal Veterinary and Agricultural University Copenhagen V*

Requests for reprints should be addressed to P Krogh Institute of Hygiene and Microbiology Royal Veterinary and Agricultural University Bülowsvej 13 DK 1870 Copenhagen V

weight in 1 and 2 days respectively The last 2 pigs died in coma with renal insufficiency and 2500–2900 μg urea per ml plasma Accordingly the creatinine content was increased and a high glucosuria was observed One pig which received 40 mg/kg for 8 days was killed because of feed refusal whereas decreased appetite was observed among the other pigs

At the level of 20 and 40 mg citrinin/kg body weight growth depression loss of weight and glucosuria was observed whereas only a slight proteinuria was present Characteristically a strong increase of blood urea and blood creatinine was observed with a maximum after one week 3 times the normal value in pigs receiving 20 mg citrinin/kg and 5 times the normal value in pigs receiving 40 mg citrinin/kg In the urine sediment a big amount of large tubular epithelial cells with large pale nuclei was observed

At necropsy and subsequent histological examination the only alterations were found in the kidneys They were enlarged as regards the pig that received 40 mg citrinin/kg during 42 days 5 times the normal size The kidneys were grey yellow with a large number of small cysts under the surface Histologically the damage is characterized by a chronic degeneration of the proximal tubules especially in the last two thirds of the convoluted portion with development of atypical basophilic epithelium with enlarged polygonated nuclei with marked chromatin

The cysts are dilated tubular segments Thickening of the tubular basement membranes activation of the interstitial cells and formation of connective tissue was also observed

In Danish pigs the kidney damage is comparable to the chronic kidney degeneration caused by feeding mouldy grain as described by *Larsen* (1928)

Thus it is assumed that the kidney degeneration naturally occuring among Danish pigs is caused by citrinin although a synergistic effect with other compounds might exist.

A full report will be published later

References 1 *Krogh P & Hasselager E* Royal Vet Agric Col Yearbook 198–214 1968 —2 *Larsen S* Månedsskr Dyrl *40* 259–284 289–300 1928

Supported by *Statens almindelige Videnskabsfond* and *Kornkvalitetsudvalget* under *Akademiet for de tekniske Videnskaber*

Acta path microbiol scand 77 561–577 1969

The Institute of Pathology II University of Umeå S 901 87 Umeå 6 Sweden the Division of Research Sinai Hospital of Detroit Detroit Mich 48235 USA and the Department of Anatomy University of Ibadan Ibadan Nigeria

SOME HISTOLOGICAL HISTOCHEMICAL AND ULTRA-STRUCTURAL STUDIES AND HORMONE ASSAYS IN A TRANSPLANTABLE ISLET CARCINOMA OF THE SYRIAN HAMSTER

By

STURE FALKMER LENNART BOQUIST PIERO P FOÀ
T ADESANYA I GRILLO DOROTHEA L BAXTER GRILLO JEAN
CLAUDE SODOYEZ FRANÇOISE SODOYEZ GOFFAUX
and ALBERT J WHITTY

Received 16 v 69

The pancreatic islets contain four types of parenchymal cells three of them are granulated (α or D α or A and β cells) and one is agranular and probably identical to the γ cell of *Bowie* (1921) On the basis of histochemical and autoradiographic studies (*Falkmer et al* 1964) and of experimental and ultrastructural investigations with various kinds of regenerating islet tissue (*Boquist* 1968 a and b *Falkmer & Bergdahl* 1967) it has been suggested that the agranular cells are precursors of the granulated cells notably of the β cells (*Boquist & Falkmer* 1969b) Agranular cells are numerous in poorly differentiated human islet cell tumours (*Greider & Elliott* 1964 *Falkmer & Bergdahl* 1967 *Boquist & Falkmer* 1969a) and according to preliminary observations (*Boquist & Falkmer* 1969b) may be identical to the non granulated or sparsely granulated cells described by *Simar et al* (1968) in a transplantable islet cell carcinoma of the hamster We thought that a more detailed study of the morphological characteristics of this tumour and of the possibility that the tumour which has been shown to produce insulin (*Grillo et al* 1967 *Sodoyez et al* 1967) may produce other biologically active substances such as pro insulin gastrin secretin mono amines and mono amine oxidase could help elucidate the physiological role of the agranular cells

Request for reprints shall be sent to Professor Sture Falkmer Institute of Pathology II University of Umeå S 901 87 Umeå 6 Sweden

This work was supported by grants from the *Swedish Medical Research Council* (Projects No K68 12X 718 03 and B69 12X 718-04A) the *Nordic Insulin Fund* and from the *National Institutes of Health United States Public Health Service* (Grants No AM06034 and FR05641)

Jean Claude Sodoyez is an Aspirant of the Fonds National de la Recherche Scientifique Belgium

MATERIALS AND METHODS

Tumour Transplants

Intramuscular and subcutaneous transplants of the tumour were prepared in adult Syrian hamsters (*Mesocricetus auratus*) as described previously (*Grillo et al* 1967) The samples of tumour tissue were excised after 3 to 9 weeks of growth from living anaesthetized animals and following the removal of grossly necrotic parts they were processed as described in each of the following sections

Light Microscopy

Specimens of tumour tissue fixed in Bouin s or Helly s fluids and embedded in paraffin were used for the common granule staining methods for islet cells and for silver impregnation by a new silver nitrate procedure (*Grimelius* 1968) and by a modification of Davenport s method (*cf Hellman & Hellerstrom* 1969) Heavy metals were detected using the sulphide silver procedure of Timm and Voigt (*Fall mer et al* 1964) in fresh frozen sections with H S and in paraffin embedded material fixed in H S saturated 70 per cent ethanol Biogenic amines were identi fied by fluorescence microscopy (*Falck et al* 1962 *Cegrell* 1969 *Cegrell et al* 1969) For this purpose pieces of tissue were lyophilized exposed to dry for maldehyde gas at 80 C for 3 hours embedded in paraffin cut into sections of 8 to 10 micra After removing the paraffin the sections were examined with a fluor escence microscope

Electron Microscopy

The tissue was prepared for electron microscopy according to the procedures described by *Boquist* (1967) and *Pihl* (1968) The former was used for conventional fine structure analysis the latter for the demonstration of heavy metals using a modification of the sulphide silver method

Atomic Absorption Spectrophotometry

The occurrence of heavy metals notably zinc and cobalt was checked by atomic absorption spectrophotometry (*cf Hau u* 1969) Biopsy specimens from the tumour the thigh muscles of the opposite side the pancreas and the myocardium were taken from each of 5 to 8 hamsters the specimens were pooled and stored at 80 C

Determination of Pro insulin[1]

About 10 g of freshly excised tumour tissue were minced blotted on filter paper packed in dry ice and mailed to Chicago where 150 mg of tumour tissue were ex tracted with acid ethanol and fractionated on a column of Sephadex G 50 medium in 1 M ac tic acid The fractions were collected in albumin coated tubes evapor ated to dryness and dissolved in a buffer consisting of 0 1 M tris HCl pH 8 1 0 05 M sodium chloride and 0 25 per cent bovine serum albumin Immuno assay of the fractions was carried out using an antiserum to crude bovine pro insulin which reacts similarly with bovine pro insulin and insulin (*cf Steiner et al* 1969)

Assay for Gastrin

Gastrin was assayed in two laboratories using slightly different methods In one laboratory four batches of pooled tumour tissue weighing 16 6 to 55 8 g and one batch of fresh hog gastric mucosa weighing 1 4 kg were treated according to the method of *Gregory & Tracy* (1961) to make extracts equivalent to 0 7–15 g of tissue per ml All steps (except boiling) were carried out in the cold The gastric se cretagogue activity of the extracts was tested in five rats using the technique described by *Lai* (1964) Each animal received injections of saline and of extracts of tumour and gastric mucosa in varying order In the other laboratory[2] about 5 g of pooled tumour tissue were collected as described above frozen at 40 C with in ½–1 minute after excision packed in dry ice and mailed to Newcastle upon

[1] Kindly performed by Dr *Donald F Steiner* Department of Biochemistry University of Chicago

[2] This gastrin assay was kindly performed by Dr *Eric I Blair* Department of Physiology University of Newcastle upon Tyne

Tyn Here two samples weighing 1 3 and 1 1 g were broken up in a loosely fitting Potter Elvehjem homogenizer rotating at 660 r p m in approximately 4 volumes of water The homogenates were placed in a boiling water bath for 3 minutes and centrifuged Aliquots of supernatant fluid equivalent to 0 3 0 9 and 1 1 g of tumour tissue were assayed for gastrin activity by measuring their ability to stimulate acid secretion by the stomach of the anaesthetized cat (*cf Blair et al* 1969) The experimental procedure was controlled using 2 0 g of tumour tissue from a case of Zollinger Ellison syndrome (*Ja Ison et al* 1963) This tumour tissue had been stored at 18 C for 5 years

Assay for Secretin[3]

Two samples of tumour tissue weighing about 5 g each were used for secretin assay In addition the small intestine of 4 anaesthetized tumour bearing hamsters was excised cut up and freed from intestinal contents adjacent fat and blood The fresh material was minced immersed within 2 minutes into vigorously boiling deionized water (about 3 g in 20 ml water) and boiled for 8–10 minutes (*Mutt* 1959) After cooling the tissue specimens were collected by filtering and resuspended in approximately 0 5 N acetic acid Glacial acetic acid was added to the filtrate in an amount sufficient to make a solution approximately 0 5 N Both fractions were saved and carried either in dry i e or at room temperature to Stockholm Here crude secretin was prepared within a week after collection of the material by adsorption on alginic acid elution with 0 2 N HCl and in some experiments precipitation with NaCl (*Mutt* 1959) The activity of the eluates was assessed in diluted aliquots (1 ml corresponding to about 0 5 or 1 0 g of wet tissue) by measuring the secretion of pancreatic juice in anaesthetized cats (*Mutt & Söderberg* 1959) A solution in physiological saline of pure porcine secretin containing 10 clinical units per ml was used as standard

Assay for Mono amine Oxidase

The presence of mono amine oxidase (MAO) was demonstrated histochemically by the reduction of tetrazolium salts (*Glenner et al* 1957) and chemically by the conversion of ^{14}C labelled 5 hydroxytryptamine (5 HT) to labelled 5 hydroxy indoleacetic acid (HIAA) according to the method of *Baker* (1966) For this purpose the tissue was homogenized (10 per cent w/v) in cold 0 1 M phosphate buffer pH 7 4 Aliquots of 0 2 ml were transferred to 5 ml centrifuge tubes kept in an ice bath 0 01 ml of labelled 5 HT creatine sulphate solution in distilled water (Radiochemical Centre Amersham specific activity 98 mc/mg 1 mg/ml) was added to each tube and the mixture was incubated at 37 C pH 7 4 for 20 minutes After incubation 0 15 ml of 1 N HCl and 3 ml of diethyl ether were added and the tubes were stoppered shaken and centrifuged at 2 00 r p m for 15 minutes at 0 C Two ml aliquots of the ether phase were pipetted into planchettes allowed to dry and their radioactivity was measured in a gas flow counter Control homogenates were heated to the boiling point cooled and incubated with the substrate

Assay for Proteolytic Enzymes

Four pieces of approximately 50 mg of tumour tissue were added to each of 20 Erlenmeyer flask containing 5 ml of Krebs Ringer bicarbonate buffer enriched with albumin (Fraction V Armour Pharmaceutical Co 0 5 per cent) and glucose (150 mg per cent) After incubation at 37 C in a Dubnoff metabolic shaker for 1 hour the pieces of tumour were removed by centrifugation the supernatant fractions were pooled and labelled insulin or labelled glucagon was added The mixture was then divided into 20 ml portions as follows a control portion containing an equal volume of a 0 5 per cent solution of albumin in phosphate buffer pH 7 4 and 5 experimental portions containing insulin (Iletin Lilly lot SG 1780-AMS) proteolytic enzyme inhibitor (Trasylol FBA Pharmaceuticals Inc) bovine albumin ACTH (Parke Davis and C # 35971) or glucagon (Lilly lot SF 8445-AMS) After standing at room temperature for various intervals of time 0 5 ml samples were removed from each flask mixed with 0 5 ml of a 10 per cent solution

[3] Kindly performed by Dr *Viktor Mutt* Gastrointestinal Hormone Research Chemistry Department Karolinska Institutet Stockholm

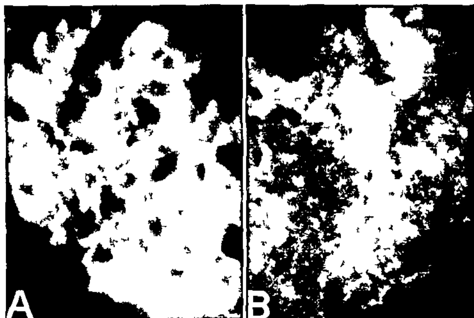


Fig 1

Green (A) and slightly yellowish (B) fluorescence of tumour tissue after exposure to formaldehyde gas indicating the presence of biogenic monoamines × 400

of trichloracetic acid (TCA) and centrifuged The precipitate was washed with an equal volume of 5 per cent TCA and its radioactivity was counted and expressed as per cent of the initial radioactivity

RESULTS

Light Microscopy

The most common tumour cells did not stain with aldehyde fuchsin showed no metachromasia with pseudo isocyanin and were devoid of secretory granules clearly identifiable by light microscopy None of the pseudo isocyanin negative cells were argyrophilic by either of the two silver impregnation methods or showed other tinctorial or histochemical characteristics of the α_1 or the α cells The second most common tumour cells were faintly aldehyde fuchsin positive contained sparse cytoplasmic granulation showing pseudo isocyanin metachromasia and for these reasons were classified as β cells No heavy metals could be detected with either variant of the sulphide silver method in the pseudo isocyanin negative cells or in the β cells

Catechol and Indole Derivatives and Mono amine Oxidase Activity

The production of a green sometimes slightly yellowish fluorescence following treatment with formaldehyde gas suggested that the tumour contained biogenic monoamines (Fig 1) From preceding chemical analyses (Cegrell et al 1969) it is known that this fluorescence es



Fig 2
Tetrazolium reduction suggesting mono amine oxidase activity A tumour tissue B pancreas of the lizard *Agama agama* islet of Langerhans surrounded by acinar tissue X 250

sentially is produced by a hitherto unidentified possibly monoamine like substance and also by dopa dopamine and 5 HT Fig 2 demonstrates the reduction of tetrazolium salts by tumour tissue and by a normal pancreatic islet and the relatively weak activity of the acinar tissue Table 1 shows that a marked MAO activity was found in tumour tissue and that this activity was almost three fold greater than that of normal hamster pancreas slightly higher than that of adult rat pancreas but lower than that found in the pancreas of rat and human foetus and of chick embryos

TABLE 1

Assay for the Activity of Mono amine Oxidase in Homogenates of Pancreas and in Islet Cell Tumour Pattern of 5 hydroxyindolea etic Acid Obtained Following Incubation with ^{14}C Labelled Serotonin (Counts/min/mg of Dry Weight)

Tissues	Untreated Homogenate	Heated Homogenate
Hamster Tumour	14 956 ± 579	1 684 ± 467 P < 0 01
Hamster Pancreas	5 034 ± 4 1	1 058 ± 287 P < 0 01
Rat Pancreas	10 562 ± 168	3 153 ± 931 P < 0 01
Pancreas of a 17 Day Chick Embryo	2 964 ± 1162	2 546 ± 461 P < 0 001
Pancreas of a 133 mm Human Foetus	20 04 ± 3698	1 769 ± 2[illegible] P < 0 01
Pancreas of a 22 Day Rat Embryo	18 956 ± 845	761 ± 273 P < 0 001



Fig 3

Low power electron micrograph of the cells of the transplantable Syrian hamster islet carcinoma, showing a predominance of agranular or only sparsely granulated tumour cells The nuclei are of varying shape sometimes with identations The cytoplasm is electron translucent Several large mitochondria and lysosomal dense bodies are seen × 3 000

Electron Microscopy

The most abundant cells were polygonal with a rather electron transparent cytoplasm and a large nucleus often showing indentations

(Figs 3 and 4) Mitochondria varied in number and were frequently abundant the Golgi apparatus was usually prominent Most of these cells seemed to lack characteristic secretory granules although several of them with otherwise identical ultrastructural features contained occasional round granules of the β type (Figs 4 and 5) Several kinds of cytoplasmic bodies were observed some of them were peculiarly shaped and apparently related to mitochondria and lysosomal dense bodies The endoplasmic reticulum was both smooth and rough and moderately prominent often forming clear round vacuoles Parasomes were rare free ribosomes moderately frequent Cells with occasional β like secretory granules sometimes contained basal bodies of cilia as well as one or more bundles of tonofilaments Except for the few β granules the ultrastructural characteristics of the cells identified as β cells by light microscopy were essentially those described by *Simar et al* (1968) No α_1 or α cells were found The modified sulphide silver procedure for detecting heavy metals gave practically negative results both in the agranular or sparsely granulated tumour cells and in the tumour cells with β granules

Atomic Absorption Spectrophotometry

The average zinc content of tumour tissue was 2 1 mg/100 g wet weight that of the 3 other hamster tissues assayed in parallel was of the same order of magnitude Likewise no cobalt content significantly higher than in the 3 other hamster tissues was observed in the tumour

Proinsulin

Two peaks of immunoreactive material with the characteristic positions of pro insulin and insulin were detected in the fractions obtained from the Sephadex column the early pro insulin peak represented 20 per cent of the total immunological activity Adsorption of the antiserum with purified bovine insulin significantly enhanced its ability to react with early peak material suggesting that this material was pro insulin or a related substance

Assay for Gastrin

Fig 6 shows that no evidence for the presence of gastrin in tumour tissue could be obtained using the first extraction procedure and the rat assay method although hog gastrin produced the expected stimulation of HCl secretion Tumour extracts prepared and assayed according to the second procedure and equivalent to 0 2 and 0 9 g of tissue showed no detectable gastrin activity The extract of 1 1 g of tissue contained the smallest activity detectable by the assay which is sensitive to 25 micromoles (or 50 nanograms) of synthetic human gastrin I (*cf Blair et al* 1969) On the other hand th

TABLE 2

Assay for the Presence of Secretin in HCl Eluates from Boiled Hamster Islet Carcinoma and Intestine Extracted with 0.5 N Acetic and Adsorbed on Alginic Acid

Order of injection	Preparation	Dose	Pancreatic response (μ eq alkali)
1	Purified porcine secretin	1 clin unit	280
2	Extract of hamster gut	1.0 ml	190
3	Physiological saline	1.0 ml	0
4	Extract of hamster gut	1.5 ml	160
5	Extract of hamster tumour	1.0 ml	40
6	Extract of hamster tumour	1.5 ml	30
7	Purified porcine secretin	1 clin unit	340

The amounts of pancreatic juice secreted by an anaesthetized cat after intravenous injection of the diluted eluates were expressed as micro equivalents of alkali

The effects of the eluates were compared with those of purified porcine secretin and the injections were made in the order indicated in the Table

The dilution was adjusted so that 1 ml corresponded to 1 g wet weight of boiled tissue

Proteolytic Enzymes Assay

Figs 7 and 8 show that labelled insulin added to the medium which had been used for the incubation of tumour tissue was degraded at a rapid rate and could no longer be precipitated by TCA Trasylol and ACTH provided relatively little protection against insulin degradation whereas bovine albumin insulin and glucagon effectively protected the labelled hormone Fig 9 shows that glucagon was also very sensitive to the proteolytic enzymes released by the tumour and was partially protected by insulin glucagon and ACTH

TABLE 3

Assay for the Presence of Secretin in the Sodium Chloride Precipitate of the HCl Eluates

Order of injection	*Preparation*	*Dose*	Pancreatic *response* (μ eq alkali)
1	Purified porcine secretion	1 clin unit	340
2	NaCl precipitate of hamster gut eluate	5 g	100
3	Same as No 2	10 g	130
4	NaCl precipitate of hamster tumour eluate	5 g	10
5	Same as No 4	10 g	0
6	Same as No 2	10 g	125
7	Same as No 1	1 clin unit	300

Estimated amount of tissue represented by the volume injected
Preparation of the eluates and other experimental conditions as in Table 1

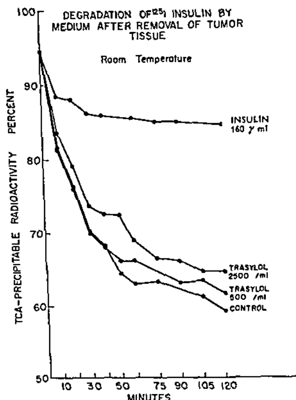


Fig 7

Degradation of 125 I labelled insulin by buffer in which tumour tissue had been incubated for 1 hour Effect of Trasylol and of non labelled insulin

DISCUSSION

The results of the light microscopic histochemical and ultrastructural investigations described in this paper confirm and extend those reported previously (*Grillo et al* 1967 *Sodoyez et al* 1967 *Simar et al* 1968) The rare occurrence of β granules in the tumour cells the negative reaction obtained with all variants of the sulphide silver procedure for the histochemical demonstration of heavy metals and the failure to find zinc or cobalt in amounts larger than those found in other tissues provide additional evidence of the low insulin content of the tumour It should be pointed out that heavy metals notably zinc and possibly also cobalt (*Havu* 1969) occur in the secretory granules of the β cells of most species (*Pihl & Falkmer* 1967) and that they probably play a role in the storage of insulin (*Falkmer & Pihl* 1968) The criteria used for the designation agranular islet cell are (*Falkmer et al* 1964 *Boquist* 1969) that the differential staining procedures for islet tissue shall give only a non characteristic background colour that the sulphide silver procedure shall be negative that the cells shall

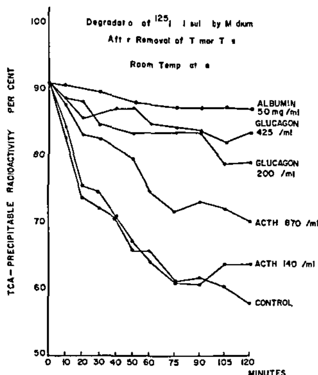


Fig 8

Degradation of ¹²⁵I labelled insulin by buffer in which tumour tissue had been incubated for 1 hour Effect of ACTH glucagon and albumin

be polygonal and have a rather prominent nucleus of varying shape sometimes with indentations and that the cells shall have an electron translucent cytoplasm and a variety of cytoplasmic organelles Usually these organelles are only moderately conspicuous but in some cases like in the cells of the hamster tumour mitochondria and Golgi apparatus are prominent the latter sometimes showing small primitive secretory granules apparently of the β type Thus the tumour appears to be constituted primarily of agranular or sparsely granulated cells and should be a good object for investigating their biological properties The presence of completely agranular cells (Fig 3) of granulated cells of the β type (Fig 5) and of an intermediate type of sparsely granulated cells with ultrastructural characteristics of both (Fig 4) supports the hypothesis that the agranular cells represent immature β cells or β cell precursors (*Boquist & Falkmer* 1969a and b *Boquist* 1969) The fact that the tumour contains less totally extractable immunoreactive insulin (*Sodoyez et al* 1967) and relatively more pro insulin than normal pancreas just as human islet cell tumours do (*Steiner et al* 1969) suggests the intriguing possibility that the pro β cells may be involved in the synthesis of pro insulin Indeed the transformation of agranular into granulated cells is very similar to

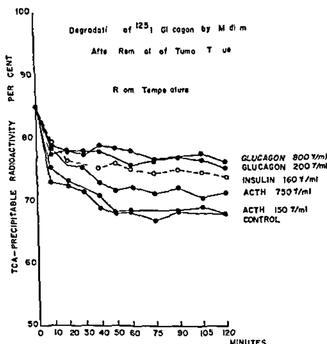


Fig 9

Degradation of ^{125}I labelled glucagon by buffer in which tumour tissue had been incubated for 1 hour Effect of ACTH insulin and non labelled glucagon

the transformation occurring in the normal islets during embryologic development (*Grillo* 1964 *Wells et al* 1968) at the time when measurable amounts of insulin begin to be synthesized (*Wessells & Rutter* 1969)

Our failure to demonstrate secretin or gastrin in tumour extracts as well as the failure to find evidence of gastrin production by the agranular cells of the islet parenchyma of lower vertebrates (*Blair et al* 1969) leaves the cellular origin of these two hormones undetermined There are however some recent reports that they may originate from granulated cells (*cf Demling & Ottenjann* 1969 *McGuigan* 1969)

The experiments described in this paper confirm the fact that the normal islets and the islet cell tumour contain biogenic amines (*Cegrell* 1969 *Cegrell et al* 1969 *Jaim Etcheberry & Ziehr* 1968 *Cession Fossion & Lefebvre* 1969) and MAO activity (*West* 1958) The role played by these substances in the pancreatic islets is unknown although they may mediate the glycogenolysis induced by anoxia (*Hellman & Idahl* 1969) the hyperglycaemic action of growth hormone and deserpidine (*Colombo et al* 1960 *Foa et al* 1953 *Galansino et al* 1960 *Sirek & Sirek* 1966) or the action of the autonomic nervous system on the pancreas The latter has been postulated on the basis of physiological (*Zunz & La Barre* 1927 *Daniel & Henderson* 1967) pharma

cological (*Kaneto et al* 1968 *Telib et al* 1968 *Cegrell* 1969 *Cegrell et al* 1969 *Nelson et al* 1967, *Kansal & Buse* 1967 *Porte* 1967) and histological (*Patent & Alfert* 1967 *Esterhuizen et al* 1968 *Morgan & Lobl* 1968) evidence The presence of biogenic amines in the hamster tumour and of MAO both in islets and in tumour tissue provides additional evidence of the similarity between this insulin producing tumour and the pancreatic islets from which it was derived The role of MAO in the biology of the tumour is a matter of speculation it is possible that the carbonyl derivative of serotonin produced by this enzymatic reaction may reduce oxygen uptake and P/O ratio (*Mahler & Humoller* 1968) thus decreasing the ratio of ATP to ADP and explaining at least in part the high rate of glycolysis characteristic of the tumour (*Sodoyez et al* 1969b)

The nature of the proteolytic enzymes released into the incubation medium by tumour tissue remains uncertain for they were not characterized biochemically Nevertheless the ineffectiveness of Trasylol a powerful kallicrein trypsin chymotrypsin inactivator (*Trautschold et al 1967*) suggests that the tumour enzymes are not related to those of the exocrine pancreas This hypothesis is supported by the fact that tumour cells do not contain zymogen granules (*Grillo et al* 1967 *Simar et al* 1968) It should be pointed out that while normal islets do not release proteolytic enzymes into the incubation medium (*Malaisse et al* 1967 *Sodoyez et al* 1969a) malignant tumours do (*Sylvén & Bois Svensson* 1965) The rapid proteolytic destruction of insulin and glucagon by tumour tissue *in vitro* may explain some of the difficulties encountered in the immunoassay of these hormones (*Sodoyez et al* 1969b *Nonaka et al* 1969)

SUMMARY

A transplantable insulin producing hamster islet carcinoma appeared to be constituted primarily of agranular or sparsely granulated cells and was consequently considered to be a good object for further investigations of the biological properties of these islet parenchymal cells Apart from agranular cells and β cells no other islet cells occurred in the tumour

The presence of sparsely granulated tumour cells with ultrastructural characteristics both of completely agranular cells and of granulated β cells supported the hypothesis that the agranular cells represent immature β cells or β cell precursors Further support for this hypothesis was obtained by the findings of less totaly extractable immunoreactive insulin and relatively more pro insulin in the tumour than in normal pancreas The zinc and cobalt contents were both insignificant

In addition to insulin and pro insulin the tumour cells were found to contain biogenic amines (catecholamines and 5 hydroxytryptamine)

and mono amine oxidase Moreover incubated tumour cells could re lease significant amounts of proteolytic enzymes

The islet carcinoma did not contain gastrin or secretin

REFERENCES

Baker P C Monoamine oxidase in the eye brain and whole embryo of devel oping *Xenopus laevis* Developm Biol *14* 267–277 1966

Blair E L Falkmer S Hellerström C Östberg H & Richardson D D Investiga tion of gastrin activity in pancreatic islet tissue Acta path microbiol scand 75 583–597 1969

Boquist L Morphology of the islets of the non diabetic adult Chinese hamster *Cricetulus griseus* Acta Soc Med Upsal *72* 345–357 1967

Boquist L Alloxan administration in the Chinese hamster I Blood glucose va riations glucose tolerance and light microscopical changes in pancreatic islets and other tissues Virch Arch Abt B Zellpath *1* 157–168 1968 a

Boquist L Alloxan administration in the Chinese hamster II Ultrastructural study of degeneration and subsequent regeneration of the pancreatic islet tissue Vich Arch Abt B Zellpath *1* 169–181 1968 b

Boquist L The endocrine pancreas in the Chinese hamster Studies on non diabetic alloxan treated zinc deficient and spontaneously diabetic animals Thesis University of Ume 1969

Boquist L & Falkmer S Agranular and ciliated parenchymal cells in normal re generating and neoplastic pancreatic islet tissue Acta path microbiol scand (in press) 1969 a

Boquist L & Falkmer S The significance of agranular and ciliated islet cells In Falkmer S Hellman B & Täljedal I B (Eds) The Structure and Me tabolism of the Pancreatic Islets A Centennial of Paul Langerhans Discovery Proceedings of an International Symposium Held in Umeå February 17–19 1969 Pergamon Press Oxford 1969 b (In press)

Bowie D J Cytological studies of the islets of Langerhans in a teleost *Neomaenis griseus* Anat Rec *29* 57–73 1924

Cegrell L Monoaminergic mechanisms in the pancreatic α cells In Falkmer S Hellman B & Täljedal I B (Eds) The Structure and Metabolism of the Pancreatic Islets A Centennial of Paul Langerhans Discovery Proceedings of an International Symposium Held in Umeå February 17–19 1969 Pergamon Press Oxford 1969 (In press)

Cegrell L Falck B & Rosengren A M Catechol and indole derivatives in a trans plantable islet cell tumour of a golden hamster Acta Physiol Scand *77* 23–27 1969

Cession Fossion A M & Lefebvre P J Catecholamines in a transplantable islet cell tumor of the golden hamster In Falkmer S Hellman B & Täljedal I B (Eds) The Structure and Metabolism of the Pancreatic Islets A Centennial of Paul Langerhans Discovery Proceedings of an International Symposium Held in Umeå February 17–19 1969 Pergamon Press Oxford 1969 (In press)

Colombo J P Weber J W Kanamoishi D & Foa P P Site of origin of a hy perglycemic substance liberated into the pancreato duodenal blood by deser pidine injections Endocrinol *67* 248–251 1960

Daniel P M & Henderson J R The effect of vagal stimulation on plasma insulin and glucose levels in the baboon J Physiol *192* 317–327 1967

Demling L & Ottenjann R (Eds) Non insulin producing tumours of the pancreas Modern aspects on Zollinger Ellison Syndrome and gastrin Proc Internat Sympos at Erlangen July 16–17 1968 Thieme Stuttgart 1969

Esterhuizen A C Spriggs T L B & Lever J D Nature of islet-cell innervation in the cat pancreas Diabetes *17* 33 36 1968

Falck B Hillarp N Thieme G & Torp A Fluorescence of catechol amines and related compounds condensed with formaldehyde J Histochem Cytochem *10* 348–354 1962

Falkmer S & Bergdahl L Ultrastructural and biological studies of the agranular cells in pancreatic islet tissue and insulomas Acta path. microbiol scand Suppl *187* 25–26 1967

Falkmer S Hellman B & Voigt G E On the agranular cells in the pancreatic islet tissue of the marine teleost *Cottus scorpius* Acta path microbiol scand. *60* 47–54 1964

Fall mer S & Pihl E Structural l ıılıty of zinc containing secretion granules of pancreatic *β* cells after exposur to hydrogen sulphide Diabetologia 4 239 243 1968

Foa P P Magil E B Glassman M H & Weinstein H R Anterior pituitary growth hormone (S11I) and pancre ı secretion of glucagon (HGF) Proc Soc Exp Biol *83* ı58–761 1953

Calansino C D Amico G K u n ıshı D Berlinger F G & Foa P P Hyper glycem c ubstances orıgın ın the pancreatoduodenal area Am J Physiol *198* 1059–1062 1960

Glenner G (Burtner H J own G B The histochemical demonstration of mon amine oxidase ı ı ıy tetrazolium salts J Histochem Cytochem 5 591 600 1957

Gregory R A & Tra y H n preparation and properties of gastrin J Physiol 156 ı 3–513 1961

Greıd r I I Flli t D Elc tron microscopy of human pancreatic tumors of islet ıı g P th ı4 663–678 1964

Grillo T A I ı ı insulin in the pancreas of foetuses of some ro dent ı — ı ı 4 1964

Grillo T ıan *H Foa P P & Kobernick S D* Biological ı r ı ı ı islet cell tumor of the golden hamster I Hı ı ı h m Diabetes *16* 409–414 1967

Grim nitrı ın for *α* cells in human pancreatic islets Acta ı 713– 1968

Hacu h ı phılıt nıl pancreatic islet tissue Experiments with al ı a ıl ı ıs chloride cadmium chloride sodium arsenite ı ı ate Acta Endocr *62* Suppl 139 1969

Hell ı ı ı lı tology and histophysiology of the islets of Lan ı n s ıf ıfer E F (Ed) Handbuch des Diabetes Mellitus J I Ichmann ı ı ı Mun hen 1969 Band I pp 89–118

Hellman B I thl I I Presence and mobilization of glycogen in mammalian pan reatic *β* cells Endocrinol *8ı* 1–8 1969

Jackson R H Blair F I Dawson P J Reed J D & Watts W P J Gastrin ac tivity f tum ur tissu in a child with the Zollinger Ellison syndrome Lan cet *II* ı 8 911 1963

Jaim Et h ı *Zı h r I M* Electron microscopic cytochemistry of 5 hy t ı HT) n the beta cells of guinea pig endocrine pancreas. En 1 –925 1 ı8

Kanetı *H Kosıla F & Nakao K* Stimulation of insulin secretion ly ı mım tıc ıgents Endocrinol *83* 651–658 1968

Kans il I *I* The effect of adrenergic blocking agents on plasma insulin a ıl bl ı cos durıng urethan or epinephrine induced hyperglycemia. Metab *16* ıs 5ıb 1967

Lai K S Studies or gastrin Gut 327–333 1964

McGuigan J I Antibodies to gastrin peptides Proc Internat Union Physiol Sci. (19ı 1969

Mahler D J & Humoller F L The influence of serotonin on oxidative metabolism of brain mitochondria Proc Soc Exp Biol Med *127* 1074–1079 1968

Malaisse W J Malaisse Lagae F Lacy P E & Wright P H Insulin secretion by isolated islets in presence of glucose insulin and anti insulin serum Proc. Soc Exp Biol Med *124* 497–500 1967

Morgan C R & Lobl R T A histochemical study of neuro insular complexes in the pancreas of the rat Anat Rec *160* 231–238 1968

Mutt V Preparation of highly purified secretin Arkiv kemi *15* 69–74 1959

Mutt V & Söderberg U On the assay of secretin Arkiv kemi *15* 63–68 1959

Nelson J K Rabinowitz D & Merimee T J Effect of epinephrine on insulin re lease in man induced by secretin Nature *215* 883–884 1967

Nonaka K Grillo T A I & Foa P P Glucagon secretion in normal rats and hamsters and in hamsters with transplantable islet cell tumor In Falkmer S Hellman B & Täljedal I B (Eds) The Structure and Metabolism of the Pancreatic Islets A Centennial of Paul Langerhans Discovery Proceedings of

an International Symposium Held in Umeå February 17-19 1969 Pergamon Press Oxford 1969 (In press)

Patent G J & Alfert M Histological changes in the pancreatic islets of alloxan treated mice with comments on B cell regen ration Acta Anat *66* 504-519 1967

Pihl E Recent improvements of the sulfide silver procedure for ultrastructural localization of heavy metals J Microscopie 7 509-520 1968

Pihl E & Falkmer S Trials to modify the sulfide silver method for ultrastructural tissue localization of heavy metals Acta Histochem 27 34-41 1967

Porte D Jr A receptor mechanism of the inhibition of insulin release by epinephrine in man J Clin Invest 46 86-94 1967

Simar L J Sodoye J C Crillo T A I Nagano T Lefebvre P J & Foa P P Biological properties of a transplantable islet cell tumor of the golden hamster III Electron microscopic studies Diabetes *17* 582-587 1968

Sirek A & Sirek O V Serotonin as the hyperglycemic substance released by growth hormone Am J Physiol *211* 1018-1020 1966

Sodoye J C Luyckx A S & Lefebvre P J Biological properties of a transplantable islet-cell tumor of the golden hamster II Insulin content of the tumor and some metabolic characteristics of the tumor bearing animals Diabetes *16* 415-417 1967

Sodoyez J C Sodoye Goffaux F & Foa P P Pathogenesis of hypoglycemia in hamsters with a transplantable islet cell tumor J Lab Clin Med 73 432-438 1969 a

Sodoye J C Sodoyez Goffaux F & Foa P P Evidence for an insulin induced inhibition of insulin release by isolated islets of Lagerhans Proc Soc Exp Biol Med *130* 568-571 1969 b

Steiner D F Clark J L Nolan C Rubenstein A H Margoliash E Aten B & Oyer P E Proinsulin and the biosynthesis of insulin Rec Progr Horm Res In press 1969

Sylven B & Bois Svensson I On the chemical pathology of interstitial fluid I Proteolytic activities in transplanted mouse tumors Cancer Res 25 458-468 1965

Telib M Raptis S Schroder K E & Pfeiffer E F Serotonin and insulin release *in vitro* Diabetologia 4 253-256 1968

Trautschold I Werle E & Zickgraf Rudel G Trasylol Biochem Pharmacol *16* 59-72 1967

Wells L J Erlandsen S L & Fguchi Y Development of pancreases and adrenals of rat embryos in organ culture Anat Rec *160* 201-205 1968

Wessells N K & Rutter W J Phases in cell differentiation Scient Amer 220 36-44 1969

West G B 5 hydroxytryptamine and hyperglycemia Nature *182* 182-183 1958

Zun E & LaBarre J De l hyperinsulinémie conse utive à l hyperglycemie provoquée par l injection de dextrose Arch Intern Physiol 29 265 1927

Acta path microbiol scand 77 578-588 1969

The Department of Neuropathology Department of Neurosurgery G and the Pharmacy Aarhus Kommunehospital The University of Aarhus Denmark

MODIFIED TECNIQUE OF MUSCLE BIOPSY

By

EDITH RESKE NIELSEN
AAGE HARMSEN and JACOB HØJGAARD

Received 21 v 69

The diagnostic significance of the muscle biopsy is constantly increasing not only in neuromuscular diseases but also within the neurosurgical field and in medical diseases e g diabetes mellitus and the collagen diseases

Muscle tissue is very sensitive to any stress Therefore the technical treatment of the biopsy is of the greatest importance to the later evaluation

The object of this work is to give a collected account of all the details concerning the technical procedures obtained on the basis of 320 muscle biopsies

Detailed descriptions will be given of the taking of biopsies the preparation of the material for light and electron microscopy and of a modification of *Koelle*s histochemical method for demonstration of the subneural apparatus and of *Coers* method for demonstration of the intramuscular nervous system

The preparation of a muscle biopsy from the taking till the finished result is of great importance Therefore all procedures were performed by trained and well qualified people

TAKING OF SPECIMENS

The muscle biopsies are taken under the usual sterile precautions The operation is generally made in local anaesthesia (10–15 ml inj lidocain noradrenalin 0 5 per cent)

To prevent artifacts in the biopsy only skin and subcutis are infiltrated with the anaesthetic not the muscles

Only on rare occasions full anaesthesia is used e g in children or in restless nervous adults

A skin incision— 6 to 8 cm long— is placed longitudinally in the extremity and the muscle fascia is split in the fibre direction for about 6 cm Thus the biopsy may be taken in the whole length of the muscle fibres and it is not necessary to ensure the localization of the end plates by electro-myography on the muscle exposed

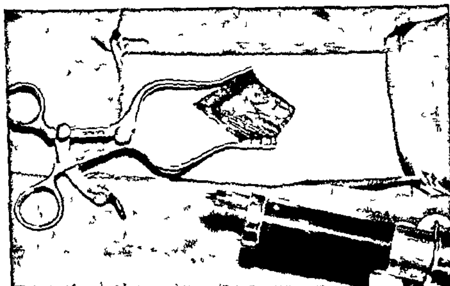


Fig 1

Biopsy of the anterior tibial muscle The fascial edges are kept apart by a self retaining retractor The exposed muscle is ready for injection of methylthionine Note the bent needle

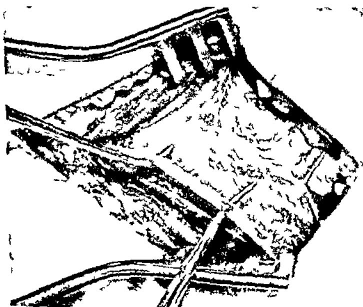


Fig 2

A muscle strip is taken for electron micr scopy by means of two single hooks

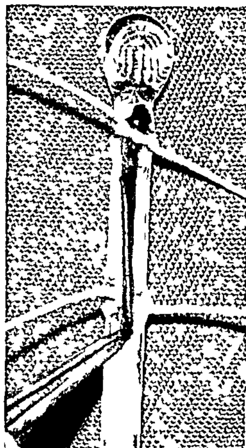


Fig 3
The muscle strip for electron micro scopy is stretched

To obtain a good result of the examination it is very important that the muscle is manipulated as little as possible

With a self retaining retractor the fascial edges are kept apart (Fig 1) and the perimysium is dissected bluntly or incised

By means of two single hooks a muscle strip (1–2 mm thick) is first taken in the entire length of the fibre for electron microscopy (Fig 2) This strip is immediately stretched on a glass rod (Fig 3) by tying a ligature in each end These ligatures are fixed on the glass rod with dental wax so that the muscle fibre may be stretched 10–15 per cent By so doing all bands in the muscle fibrils stand out very clearly in the electron microscope The glass rod with the biopsy is placed in the fixative After the taking of the muscle for electron microscopy the exposed part of the selected muscle is infiltrated for a width and depth of 1 cm by 10 20 ml inj methylthionini chloridi 0 15 per mille

The dye is injected by a thin (gauge 14) bent needle inserted in the fibre direction at various depths The injection is made slowly and carefully both during the insertion and withdrawal of the needle until the muscle is maximumly blue (Fig 1)

5 minutes after the beginning of the injection the muscle piece in question is cut distally (¾–1 cm in the width and in the depth), carefully gripping the distal end of the muscle piece with a pair of tweezers and isolating it to the proximal end Only the ends of the muscle piece must be gripped With a pair of scissors 4–5 strips are cut from the whole length of the muscle piece and placed on gauze moistened with saline for demonstration of the intramuscular nervous system One strip is placed in saline 0 9 per cent for demonstration of the subneural apparatus and one strip also placed in saline for light microscopy

TREATMENT OF MUSCLE BIOPSY

With minimal modifications earlier described methods have been used

Careful treatment of the biopsy for paraffin wax sectioning is very important to avoid shrinking and artifacts The following procedure is employed

1 day the muscle biopsy received in saline 0 9 per cent is placed on a cork with porcupine bristles (not metal needles) and stretched to its original length It remains in saline for about 2 hours, then in formalin solution 4 per cent over night

2 day the biopsy is removed from the cork washed in running water for about 8 hours and then placed in aethanol 70 per cent over night

3 day the biopsy is placed in aethanol 96 per cent which is changed once during the day

4 day the biopsy is placed in aethanol 99 per cent in a thermostat 37 C for about 8 hours and then in ligroine over night

5 day the specimen is cut transversely of fibres The two strips are placed in pure ligroine where they remain for about 4 hours then in paraffin wax in a 58 C hot oven

6 day embedded so that one piece is cut transversely the other longitudinally

7 day the specimen is cut at 7 μ and stained

The following general stainings are made on the paraffin wax section haematoxylin eosin method, Van Gieson Hansen and toluidine blue The following special stainings are made PAS Weil and Davenport (*Romeis* 1948)

According to the experience of the department the slow dehydration of the specimens and the clearing in ligroine are very important for obtaining good specimens

DEMONSTRATION OF THE SUBNEURAL APPARATUS

The principle of the histochemical method is the exploitation of the content of acetylcholine esterase in the subneural apparatus (*Bark.* ... *Anderson* 1963)

The histochemical staining of the subneural apparatus is perfor[illegible] by a simplified and modified technique described by *Koelle & Fried[illegible]wald* (1949) modified by *Couteaux* (1951)

On the basis of *Koelle*'s method two stable solutions A and B have been [illegible]pared and these two form the Incubating Medium [illegible]

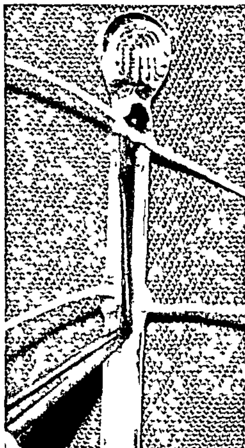


Fig 3
The muscle strip for electron microscopy is stretched

To obtain a good result of the examination it is very important that the muscle is manipulated as little as possible

With a self retaining retractor the fascial edges are kept apart (Fig 1) and the perimysium is dissected bluntly or incised

By means of two single hooks a muscle strip (1–2 mm thick) is first taken in the entire length of the fibre for electron microscopy (Fig 2) This strip is immediately stretched on a glass rod (Fig 3) by tying a ligature in each end These ligatures are fixed on the glass rod with dental wax so that the muscle fibre may be stretched 10–15 per cent By so doing all bands in the muscle fibrils stand out very clearly in the electron microscope The glass rod with the biopsy is placed in the fixative After the taking of the muscle for electron microscopy the exposed part of the selected muscle is infiltrated for at width and depth of 1 cm by 10–20 ml inj methylthionini chloridi 0 15 per mille

The dye is injected by a thin (gauge 14) bent needle inserted in the fibre direction at various depths The injection is made slowly and carefully both during the insertion and withdrawal of the needle until the muscle is maximumly blue (Fig 1)

5 minutes after the beginning of the injection the muscle piece in question is cut distally (¾–1 cm in the width and in the depth) carefully gripping the distal end of the muscle piece with a pair of tweezers and isolating it to the proximal end Only the ends of the muscle piece must be gripped With a pair of scissors 4–5 strips are cut from the whole length of the muscle piece and placed on gauze moistened with saline for demonstration of the intramuscular nervous system One strip is placed in saline 0 9 per cent for demonstration of the subneural apparatus and one strip also placed in saline for light microscopy

TREATMENT OF MUSCLE BIOPSY

With minimal modifications earlier described methods have been used

Careful treatment of the biopsy for paraffin wax sectioning is very important to avoid shrinking and artifacts The following procedure is employed

1 day the muscle biopsy received in saline 0 9 per cent is placed on a cork with porcupine bristles (not metal needles) and stretched to its original length It remains in saline for about 2 hours then in formalin solution 4 per cent over night

2 day the biopsy is removed from the cork washed in running water for about 8 hours and then placed in aethanol 70 per cent over night

3 day the biopsy is placed in aethanol 96 per cent which is changed once during the day

4 day the biopsy is placed in aethanol 99 per cent in a thermostat 37 C for about 8 hours and then in ligroine over night

5 day the specimen is cut transversely of fibres The two strips are placed in pure ligroine where they remain for about 4 hours then in paraffin wax in a 58 C hot oven

6 day embedded so that one piece is cut transversely the other longitudinally

7 day the specimen is cut at 7 μ and stained

The following general stainings are made on the paraffin wax section haematoxylin eosin method Van Gieson Hansen and toluidine blue The following special stainings are made PAS Weil and Davenport (*Romeis* 1948)

According to the experience of the department the slow dehydration of the specimens and the clearing in ligroine are very important for obtaining good specimens

DEMONSTRATION OF THE SUBNEURAL APPARATUS

The principle of the histochemical method is the exploitation of the content of acetylcholine esterase in the subneural apparatus (*Barka & Anderson* 1963)

The histochemical staining of the subneural apparatus is performed by a simplified and modified technique described by *Koelle & Friedenwald* (1949) modified by *Couteaux* (1951)

On the basis of *Koelle*s method two stable solutions A and B have been prepared, and these two form the Incubating Medit

Solution A is composed in the following way

Sodium acetate (CH_3COONa $3H_2O$)	3 320 g
1 N acetic acid	0 125 ml
Demineralized water to make	250 ml

pH is tested to 6 20 after which the solution is mixed with a solution consisting of

Cupri sulphate ($CuSO_4$ $5H_2O$)	250 mg
Aminoacetic acid	375 mg
Demineralized water to	210 ml

Solution A consists of *Koelle*s solution 1 + 2 + 3

Solution B also named solution medium consists of

Cupri sulphate ($CuSO_4$ $5H_2O$)	1 248 g
Demineralized water to make	200 ml

In a refrigerator (4 C) the solutions A and B will keep for 3 months

Before the incubating solution shall be prepared a sufficient amount of the solutions A and B is heated to 20 C–22 C

Incubating Solution

145 mg of ATHCH (acetylthiocholiniodide) is dissolved in 10 ml of solution B and centrifuged for about 20 minutes The supernatant is filtrated and 2 4 ml of the clear filtrate is mixed with 27 6 ml of solution A (must be prepared fresh day by day)

By this modification two stable liquids are obtained (solution A and solution B) which may be prepared at the local pharmacy as well as a stable substance ATHCH which will remain stable provided they are kept under proper conditions Thus the technical staff has only two solutions and one dry solid to work with which is a great relief

Subsequently the procedure is a follows

1 The muscle biopsy is kept in saline 0 9 per cent for one hour
2 Then in neutral 10 per cent formaldehyde solution for 1–2 hours
3 Longitudinal sectioning on freezing microtome 60–70 μ thick
4 Rinsed twice in distilled water
5 The sections are incubated at 37 C in the incubating solution At this enzymic reaction a copper thiocholine precipitate is formed and at every 15 minutes sections are taken out These go through the following procedure rapidly
6 Rinsed twice in distilled water
7 Placed in a 5 per cent ammonium sulphide solution for 1–1½ minute

By so doing the copper thiocholine precipitate is transformed into an amorphous copper sulphide by which the end plates become visible (The above procedure is continued until a suitable colour intensity is obtained)

8 The specimens are rinsed twice in distilled water
9 Dipped briefly into 3 per thousand toluidine blue (*F Christensen* 1959)
10 The sections are now mounted dehydrated and embedded

The sections are during the whole procedure manipulated with a glass hook

This modification of *Koelle s* staining is easily performed and renders excellent results (Fig 4)

Vital Staining of the Intramuscular Nervous System with Methylene Blue

The demonstration of the intramuscular nervous system is initiated with the intravital staining with methylthionine chloride The dye is

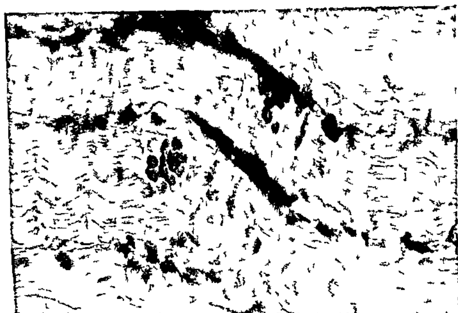


Fig 4
Normal subneural apparatuses in a anterior tibial muscle

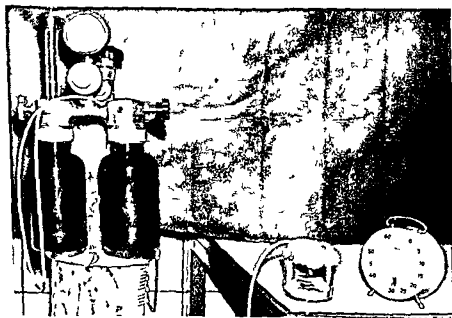
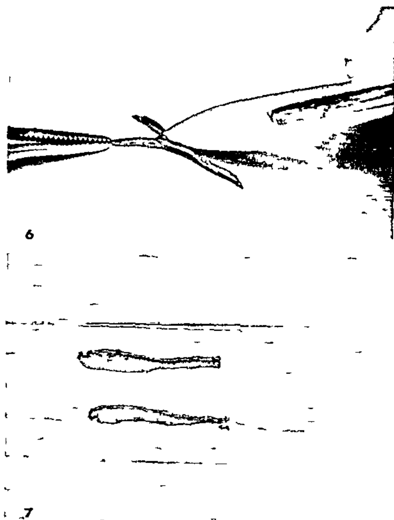


Fig 5
The muscle strips are placed under cover and oxygenated



Figs 6–7

Fig 6 A muscle strip is split longitudinally by a loose leaf scalpel
Fig 7 Muscle strips are squeezed between two slides

bound in the intramuscular nervous system and colours this At after treatment of the biopsy with oxygen the existing leucomethylthionine derivatives will be transformed into methylthionine derivatives

The preparation of the biopsy after the taking is a modification of *Coers* method (*Coërs and Woolf* 1959 *Coers* 1952a)

The procedure is performed in the following way

1 The muscle strips are placed on moistened gauze under cover and frequently sprinkled with saline 0 9 per cent The specimens are oxygenated for one hour in pure oxygen with a suitable flow (after 30 minutes the specimens are turned) (Fig 5)

2 The strips are now stretched to the original length on a cork with porcupine

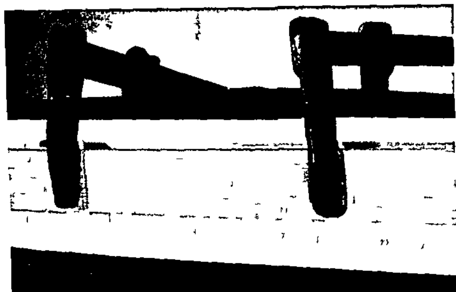


Fig 8
The slides are pressed between two even wooden pieces

bristles and fixed in a filtered cold aqueous saturated solution of ammonium molybdate at 4 C for twenty four hours

3 Then washed in three changes of distilled water 4 C

4 The strips are then split longitudinally by hand by a loose leaf scalpel at 30 μ placed between two slides and squeezed carefully (Fig 6 and 7)

5 The slides are kept in press between two even wooden pieces (Fig 8) by means of two screw clamps during about one hour

6 The slides are carefully separated

7 Manual dehydration of the specimens on the horizontally placed slides by means of a pipette

Twice for about 2 minutes in aethanol 70 per cent
Twice for about 2 minutes in aethanol 96 per cent
Twice for about 2 minutes in aethanol 99 per cent

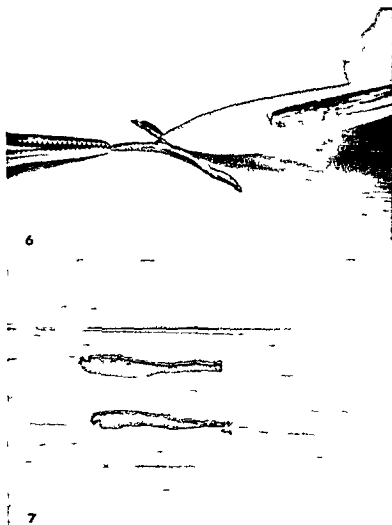
8 Clarification in toluene twice Then embedding in resin The hand made specimens render an extremely beautiful result with possibility for study as well as photography of the single end plates and of fibres and bundles

Contrary to *Coers* method hand cut preparations are used which give a good survey of bundles as well as of terminal axons In the last five cases the muscle strip is stretched after fixation with good result (Fig 9 A + B) According to our opinion this is more careful with the tissues than freeze cutting The simultaneous pressing of all preparations is a facilitation to the technical staff

The Preparation of Muscle Specimens for Electron Microscopy

The following solutions are needed

1 Fixative (*Pease* 1964)
4 ml of glutaric acid 25 per cent



Figs 6–7

Fig 6 A muscle strip is split longitudinally by a loose leaf scalpel
Fig 7 Muscle strips are squeezed between two slides

bound in the intramuscular nervous system and colours this At after treatment of the biopsy with oxygen the existing leucomethylthionine derivatives will be transformed into methylthionine derivatives

The preparation of the biopsy after the taking is a modification of *Coers* method (*Coers and Woolf* 1959 *Coers* 1952a)

The procedure is performed in the following way

1 The muscle strips are placed on moistened gauze under cover and frequently sprinkled with saline 0 9 per cent The specimens are oxygenated for one hour in pure oxygen with a suitable flow (after 30 minutes the specimens are turned) (Fig 5)

2 The strips are now stretched to the original length on a cork with porcupine

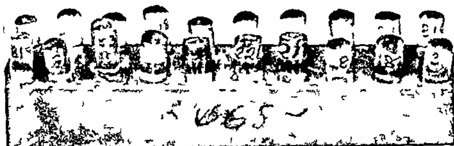


Fig 10

Capsules with imbedded specimens are placed in a plastic rack

4 Vestopal W
100 ml of Vestopal
1 ml of initiator
1 ml of activator

The Vestopal is stirred well with 1 ml of initiator for 5 minutes then 1 ml activator is added and the mixture is stirred well for 5 minutes

The preparation of the specimens for electron microscopy is then as follows

1 Fixation in glutaric aldehyde for 24 hours

2 Rinsing fluid for 24 hours

3 The muscle biopsy is removed from the glass rod the middle piece with the assumed end plates is divided into thin layers and further fixed in osmium tetroxide for one hour

4 Rinsing fluid for 24 hours

5 Dehydration

20 minutes in aethanol 24 per cent
20 minutes in aethanol 60 per cent
20 minutes in aethanol 62 per cent
20 minutes in aethanol 93 per cent
2 × 15 minutes in aethanol 99 per cent
2 × 45 minutes in 100 per cent acetone p a with dried copper sulphate (filtered before use)

6 The strips are placed in a beaker with a mixture of Vestopal 25 per cent and pure anhydrous acetone 75 per cent p a They remain uncovered in this solution for 24 hours in a thermostat at 30 C

7 As much as possible of the Vestopal is poured off and the beaker is filled with an identical amount of 100 per cent Vestopal This is left uncovered at 30 C for 24 hours

8 Embedding The specimens are placed at the bottom of small gelatine capsules by means of a thinly drawn out glass rod a small label with number and consecutive letters are placed inside at the top of the capsule with a pair of fine tweezers Then the capsules are filled with Vestopal by a normal eye pipette with cut off point

The capsules are placed in a plastic rack which may hold 20 capsules in all (Fig 10)

9 The specimens are placed in the oven for 24 hours at 30 C
24 hours at 40 C
48 hours at 60 C

10 Filing

The specimens may be cut 24 hours after the final preparation

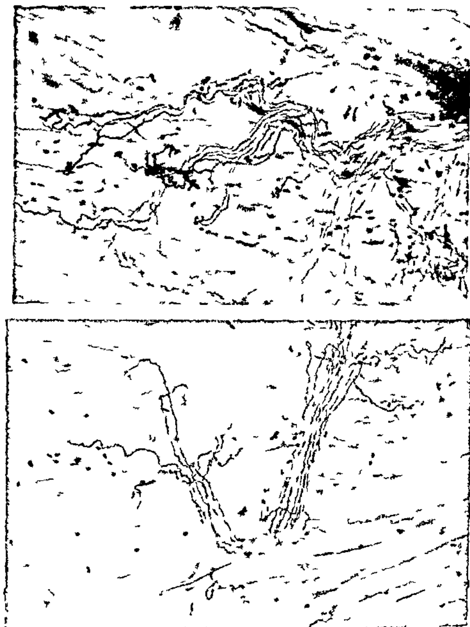


Fig 9 A and B

Nervebundles subterminal fibres and end plates in a stretched normal long palmar muscle A Low magnification B High magnification

20 ml of phosphate buffer pH 7.3
20 ml of distilled water
The ingredients are mixed well immediately before use
2 1 per cent isotonic osmium tetroxide solution (0 9 per cent saline)
3 Rinsing fluid 0 15 M *sucrose in* 0 075 M *phosphate buffer* pH 7 3

Acta path. microbiol scand 77 589–597 1969

The Departments of Pathology II and Orthopaedic Surgery University of Goteborg Goteborg Sweden

CLEAR-CELL SARCOMA OF TENDONS

A Study of 4 Cases

By

Lennart Angervall and Bertil Stener

Received 23 vi 69

Malignant tumours arising from tendons are rare Among those reported most have been interpreted as synovial sarcoma or fibrosarcoma until *Enzinger* in 1965 described a new type which he called clear cell sarcoma of tendons and aponeuroses This tumour which is roughly spherical firm and usually well defined consists of compact nests and fascicles of pale staining round or fusiform cells of epithelioid appearance The cellular aggregates are enclosed by delicate fibrous septa merging with tendinous or aponeurotic tissue Most of the 21 tumours studied by *Enzinger* were located in the lower extremity the foot being the most common site The tumours had been collected in the files of the Armed Forces Institute of Pathology in Washington D C USA during 25 years Considering the large material of this institute these figures reflect the rarity of the tumour Additional information on clear cell sarcoma is given in the present paper The 4 tumours studied by us had been collected in the Departments of Pathology Sahlgren Hospital Goteborg during the last 20 years again an indication of the rarity of the tumour as these departments collect material from a part of Sweden inhabited by more than 1 million people

CASE REPORTS

Case 1 A man 39 had received a hard blow to the inner aspect of the left knee 2 years before he presented with a tumour in the same region During the last 3 months the tumour had increased rapidly in size and had become painful.

On examination a firm non fluctuant rounded tumour was felt posteromedial to the knee joint The circumference over the most prominent part of the tumour was 46 cm as compared with 34 cm on the other leg The mobility of the joint was greatly restricted The skin over the tumour felt warm Several enlarged lymph nodes (2–3 cm) were palpated in the left groin

An incisional biopsy (December 1950) was done and the pathologist s report was a synovial sarcoma Radiotherapy was given to the primary tumour (tumour dose 4000 R) without any decrease of its size and to the regional lymph nodes (skin dose 3300 R) with some regression as a result The patient died after 6

We are indebted to Professor *Ingmar Wickbom* for interpretation of the radiographic findings in Cases 3 and 4 and for allowing us to publish Fig 1

months with radiographic evidence of metastatic lesions in the lungs the lumbar spine and the pelvis

Case 2 A man 27 had noticed a slow growing painless mass in the right lower leg for 3 years

Examination revealed a tumour 7 × 5 cm b hind the tibial malleolus The tumour moved with the Achilles tendon during flexion and extension of the ankle joint

At operation (November 1960) the tumour was found to lie close to the Achilles tendon It was excised together with the overlying skin and the adjacent part of the tendon

Two years and 3 months after the operati n a lymph node enlargement 3 × 5 cm suspected to be a metastasis was *palpated in the right groin A slight re*gression was noticed following radiotherapy (skin dose 1500 R) An ilio inguinal lymph node dissection was then carried out Histological examination confirmed metastatic lesion in the palpated enlarged node

Three years after the excision of the primary tumour the patient died with metastatic lesions in retroperitoneal and mediastinal lymph nodes the skin the lungs the pleura the myocardium the pericardium the liver the peritoneum the omentum and the skeleton

Case 3 A man 28 had noticed a growing lump near the upper inner margin of the right patella for 1 year He had experienced pain in the lump while working with the knee bent

Examination showed a firm ovoid tumour 7 × 5 × 5 cm locate l at the musculotendinous junction of the vastus medialis The tumour moved with the patella on flexion and extension of the knee joint

Femoral angiography (Fig 1) showed that the tumour was highly vascular Many irregular tortuous vessels filled within the tumour some of them varying markedly *in width moreover a diffuse opacification by contrast medium oc*curred Veins draining the tumour filled while contrast medium still remained in the femoral artery indicating arteriovenous shunting Angiographically the tumour appeared well defined

At operation (January 1961) the tumour was removed by a wide local excision including the whole of the vastus medialis part of the quadriceps tendon the upper medial third of the patella the fibrous capsule of the knee joint on the medial side with underlying synovial tissue and the long tendon of the adductor magnus The tibial end of the sartorius was attached to the patella in order to prevent lateral dislocation Through this procedure the distal third of the sartorius was given a direction similar t that of the removed vastus medialis

After the operation the patient returned to work as a plater At follow up 1 year later he could extend the knee joint fully and flex it 70 The transferred sartorius muscle became activated during voluntary flexion of the knee joint but remained relaxed during extension The patient had not noticed any tendency of lateral dislocation of the patella nor could such be demonstrated at clinical examination

Two years after the excision of the primary tumour iliac and lumbar lymph node metastases were removed by a retroperitoneal dissection Three months later the patient died with metastatic lesions in the skull the myocardium the pericardium and the right suprarenal gland

Case 4 A man 60 had noticed a growing mass anteriorly in the left lower leg for 2 months

On examination a firm tender tumour 5 × 3 × 3 cm was palpated in the anterior muscle compartment of the lower leg 20 cm distal to the knee joint The tumour was free from the skin and the skeleton It moved during isotonic contraction of the tibialis anterior but not during such contraction of the extensor hallucis longus and the extensor digitorum longus

Angiography revealed that the anterior tibial artery was displaced posteriorly in a smooth curve by the tumour but no abnormal vessels were demonstrated nor any arteriovenous shunting

At operation (August 1966) the tumour was found t be locate l t the musculotendinous junction of the tibialis anterior (Fig 2 arrow) This muscle



Figs 1–2

Fig 1 Cas 3 Femoral angiography demonstrating highly vascular tumour Veins draining the tumour (white arrow) have been filled while contrast medium still remains in the femoral artery (black arrow) indicating arteriovenous shunting

Fig 2 Case 4 Surgical specimen consisting of tibialis anterior with tumour (arrow) at the musculotendinous junction Note ramifying septa on the cut surfac of the tumour Scale in centimeters

was extirpated together with its fascia Tumour tissue was not exposed during the operation

At follow up examination 2 year and 2 months after the operation there were no signs of recurrence or metastasis

PATHOLOGY

Gross papearance The tumours were roughly spherical or ovoid firm solid rather well demarcated and intimately associated with a tendon in Case 1 presumably (cf Fig 3) one or several tendons passing the posteromedial region of the knee joint (medial head of gastrocnemius semimembranosus semitendinosus and gracilis) in Case 2 the Achilles tendon in Case 3 the tendon of the vastus medialis and in Case 4 the tendon of the tibialis anterior

The cut surface was grey or brownish grey In Case 4 distinct ramifying septa dividing the tumour could be recognized in the cut surface (Fig 2) The largest diameter of the tumour was 7 7 and 5 cm in Cases 2 3 and 4 respectively In Case 1 the tumour itself could not be measured but the circumference of the affected knee was enlarged by 12 cm at the time of biopsy

Histological methods The operative specimens were fixed in 10 per cent formaldehyde solution and embedded in paraffin Five μ thick sections were routinely stained according to the haematoxylin van Gieson method and with haematoxylin and eosin Laidlow s silver impregnation was used for demonstration of reticulin fibres and Weigert s elastin method for studying elastic tissue of tumour vessels Alcian blue and toluidin blue stains were used at pH 4 0 and 0 5 for examination of acid mucopolysaccharides these stainings were done with and without prior treatment of the sections with testicular hyaluronidase (Sigma) The periodic acid Schiff reaction (McManus) was done with and without prior treatment of the sections with diastase (Merck) The tumour cells were examined for cross striation as in rhabdomyoblasts with Masson s trichome staining and Heidenhein s iron haematoxylin staining For demonstration of fat the Sudan Black B method (0 1 per cent solution) was used on paraffin sections and the Scharlach R method on frozen sections (no material available for frozen sections in Cases 1 and 4) Pigment in tumour tissue was analysed with the Prussian blue reaction for iron and reducing properties as in melanin were studied with Masson s and Schmorl s methods

Microscopic appearance (Figs 3-10) All the tumours had a similar strikingly homogenous appearance characterized by round or spindle shaped pale staining epithelioid cells arranged in compact nests and fascicles well defined by septa of fibrous connective tissue The tumour cells exhibited a clear vacuolated or finely granulated cytoplasm with frequently an ill defined border The round or ovoid vesicular nuclei had a large centrally located basophilic deeply staining nucleolus in most of the cells A few multinucleated giant cells with uniform nuclei were demonstrated in Cases 2 3 and 4 Mitoses were scarce or absent The delicate septa of fibrous connective tissue defining the smallest cellular aggregates were continous with coarser fibrous septa transversing the tumour and merging with preformed tendinous tissue (Figs 3 and 8)

Mucoid extracellular material found in Cases 3 and 4 stained positively with Alcian blue and metachromatically with toluidin blue at pH 4 0 but not at pH 0 5 At the former pH the reactions were negative after prior treatment of the sections with testicular hyaluronidase Varying amounts of intracellular diastase sensitive PAS positive material were demonstrated in all 4 tumours suggesting the presence of glycogen Masson s trichrome and Heidenhein s iron haematoxylin

---

Figs 3 6

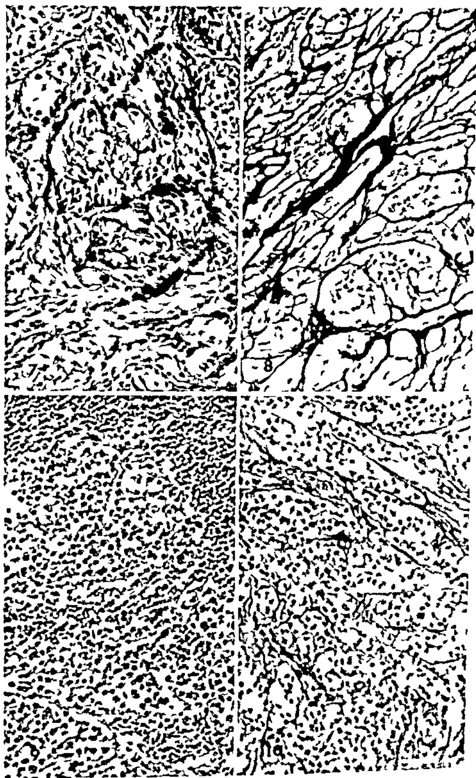
Fig 3 Case 1 Delicate septa of fibrous connective tissue defining the smallest cellular aggregates (cf Fig 4) are continuous with coarser fibrous septa transversing the tumour and merging with tendinous tissue (top) Laidlow stain × 30

Fig 4 Case 1 The tumour is composed of groups of round and spindle shaped sparsely granulated or vacuolated cells with prominent nucleoli *H and E* × 300

Fig 5 Case 2 Section from the tumour showing vacuolated fusiform tumour cells arranged in nests and fascicles H and E × 190

Fig 6 Case 2 Section from another part of the tumour showing a picture similar to that in Fig 4 Arrow indicates giant cell H and E × 190


3
5
6


7
8
9
10

stains did not reveal any cross striation of the tumour cells suggesting a myogenic origin No certain intracellular fat was demonstrated Pigment was found particularly in the fibrous septa It gave positive iron reaction indicating the presence of haemosiderin but negative reactions for melanin

The metastases to the lymph nodes in Cases 2 and 3 displayed a less orderly cellular arrangement (with loss of cellular cohesion) a more granulated and less vacuolated cytoplasm and a more marked cellular pleomorphism than did the primary tumour (Fig 9) (In Case 1 the lymph node metastases were not available for study)

Vessels with a diameter of roughly 50–150 microns were chiefly seen in the coarse fibrous septa Many of them displayed elastic tissue and smooth muscle cells in the wall but it was not possible to distinguish between afferent and efferent tumour vessels Few thin walled vessels of capillary type could be recognized within the nests and fascicles of tumour cells in Cases 1 2 and 4 In Case 3 the tumour was conspicuously vascular with many vessels of capillary or embryonal type in the nests and fascicles of tumour cells

DISCUSSION

The morphological appearance of the 4 described tumours was characterized by (1) intimate association with a tendon (2) pale staining vacuolated of finely granulated cells with prominent nucleoli and (3) cellular arrangements in nests and fascicles well defined by septa of fibrous connective tissue This morphological appearance agrees closely with that of clear cell sarcoma of tendons and aponeuroses as described by *Enzinger*

Earlier this tumour has been interpreted in several instances as synovial sarcoma and this was in fact the diagnosis in Cases 1 and 2 on the histological examination However the above mentioned characteristic morphological features and the lack of pseudo acinar structures or other forms of biphasic cellular differentiation help to distinguish clear cell sarcoma from synovial sarcoma These tumours also differ as to the staining qualities of the secreted mucoid material (*Enzinger*)

In Case 3 the tumour was first interpreted as a fibrosarcoma However the peculiar cellular features and arrangements and the low degree of desmoplastic activity are not suggestive of a fibrosarcoma (*Enzinger*)

In Case 4 an embryonal or alveolar rhabdomyosarcoma was su

Figs 7–10

Fig 7 Case 3 Vacuolated round and fusiform tumour cells arranged in small nests defined by septa of fibrous connective tissue H and E × 190

Fig 8 Case 3 Delicate ramifying septa of argentaffin fibres enclosing nests of tumour cells Laidlow stain × 190

Fig 9 Case 3 Lymph node metastasis (surgical specimen) Note the dissociation of the tumour cells which show a granulated cytoplasm and pleomorphism H and E × 190

Fig 10 Case 4 Vacuolated mostly round and uniform tumour cells arranged in small nests and fascicles separated by fibrous connective tissue H and E × 190

spected on the initial histological examination However none of the 4 tumours displayed marked acidophilia sarcolemma fibrils or cross striation suggesting rhabdomyoblasts

In some instances of *Enzinger s* series, the tumour had been confused with a malignant melanoma It is evident that nests and fascicles of spindle shaped cells may have a nevoid feature with some resemblance to that of malignant melanoma of the spindle cell type In no instance however could melanin be demonstrated with different methods neither by *Enzinger* nor by us

The tumours hardly displayed any morphological features of extraskeletal chondromatous tumours nor could sulphated mucopolysaccharides be demonstrated by staining with Alcian blue and toluidine blue at pH 0 5 The results of the mucopolysaccharide stainings combined with digestion by testicular hyaluronidase indicate the presence of non sulphated mucopolysaccharides such as hyaluronic acid in the mucoid material of the tumours As mucoid material with similar staining properties can be found extracellularly in areas of mucin degeneration in other soft tissue tumours this finding seems to be of slight significance in the diagnosis of clear cell sarcoma of tendons and aponeuroses

Vascular structures were scarce in the tumours studied by *Enzinger* One of our tumours however was highly vascular as demonstrated both angiographically (Fig 1) and histologically This tumour (Case 3) in contradistinction to the others contained many thin walled vessels of capillary or embryonal type in the nests and fascicles of tumour cells In Case 4 angiography gave the topographic information that the anterior tibial artery had been displaced by the tumour but no newley formed vessels were demonstrated

In all 4 of our patients the tumour was located in the lower extremity In *Enzinger s* series 17 out of 21 tumours had this location The mean age of the patients at surgery was 26 years in *Enzinger s* material with only 3 patients being more than 50 years old Three of our patients were between 27 and 39 years old one was 60 Our patients were men In *Enzinger s* series 8 were men and 12 were women

Among 19 patients in *Enzinger s* series on whom follow up information was available 14 had died 6 within 3 years of treatment 8 after a more protracted course lymph node metastases were observed in 7 of the deceased patients Five of the 19 patients with follow up data were alive and well 2–4 years after the last surgical procedure Three of our patients died within 3 years of treatment all with metastases to lymph nodes The fourth patient is alive and well less than 3 years after excision of the tumour

The 25 instances of clear-cell sarcoma of tendons and aponeuroses hitherto described clearly demonstrate that the tumour is malignant In fact no case of 5 year survival after the last occasion of treatment

has yet been reported It appears that one factor behind the poor result of treatment obtained so far might have been a failure to recognize the ominous nature of the lesion on the first occasion of treatment In 5 patients in *Enzinger's* series amputation was resorted to only when one or several local operations had proved to be inadequate all these patients died In 6 others who died more than one local operation was done suggesting that the initial procedure had not been radical enough When planning an operation with a view to completely removing a clear cell sarcoma of tendons the propensity of the tumour to metastasize to lymph nodes should be taken into consideration Perhaps a higher cure rate can be obtained if the regional lymph nodes are removed along with the tumour preferably in a monoblock procedure

SUMMARY

Four cases of clear cell sarcoma of tendons are presented This is a rare malignant tumour described by *Enzinger* in 1965 Our tumours occurred in men 27 28 39 and 60 years old all tumours were located in the lower extremity and were closely associated with tendinous tissue The 3 younger patients died with multiple metastases within 3 years of treatment all with metastases to lymph nodes The older man is still alive without signs of recurrence or metastasis 2 years and 2 months after excision of the tumour

Differential diagnosis is discussed on the basis of *Enzinger's* and our own morphological observations Angiographic and histological examinations indicate that clear cell sarcoma can be either poorly or highly vascular The propensity of the tumour to metastasize to lymph nodes should be taken into consideration when planning the treatment

REFERENCE

Enzinger F M Clear cell sarcoma of tendons and aponeuroses An analysis of 21 cases Cancer *18* 1163–1174 1965

Acta path microbiol scand 77 598–608 1969

Ullevål Hospital Department of Pathology (Head Kristen Arnesen M D)
University of Oslo Oslo Norway

CUSHION LIKE INTIMAL LESIONS IN INTRAMYOCARDIAL ARTERIES OF MAN

Their Relation to Age, Sex, Coronary Atherosclerosis and Certain Diseases

By

JØRGEN W HÆREM

Received 13 v 69

In transverse sections of intramyocardial arteries of man cushion like intimal thickenings are frequently seen They are characterized by subendothelial deposits of a structureless material

In a previous autopsy study the morphology of these lesions was examined in order to explore their pathogenesis (6) The most likely explanation of the lesions was that they are the results of haemodynamic trauma against the vessel wall

In the present investigation further information about the pathogenesis of the intimal cushion like lesions is obtained by studying associations between the lesions and age sex coronary atherosclerosis and several diseases present in the autopsy series

MATERIAL AND METHODS

The consecutive autopsy series was the same as in the former study (6) It consisted *of 129 males and 86 females from 1 to 96 years of age (Fig 1)* One hundred and twenty two men and 84 women were 40 years of age and older the median age of men and women was 65 and 73 years respectively

The autopsies were performed 8 to 34 hours after death A detailed description of the sampling of the myocardium the processing of histological sections and of staining methods has already been given (6)

Microscopical screening with a magnification of 100x was performed in all hearts of one section from the interventricular septum and of one from the lateral wall of the left ventricle In randomly selected cases one section from other parts of the ventricle also was screened All clinical and autopsy data were unknown during the microscopical examination Sections containing recent or old infarcts were not screened

As the cushion like intimal lesions rarely were found in arteries larger than 150 microns or in arterioles smaller than 30 microns in diameter only arteries within these limits were counted They were [illegible] red as having or not having intimal

---

Requests for reprints should be addressed to *Jørgen W Hærem* Ullevål Hospital Department of Pathology Oslo 1 Norway

This work has been supported by grants from the *Norwegian Council on Cardiovascular Diseases* and from *J L Tiedemanns Tobaksfabrik – Johan H Andresens medicinske fond* *Leif Jørgensen* M D has given help and advice

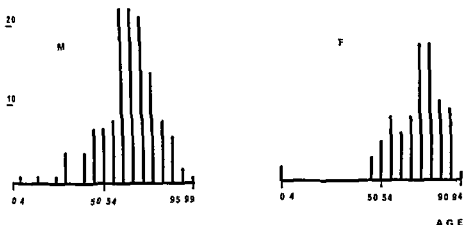


Fig 1

Diagram showing the age distribution of the 1?9 males (M) and the 86 females (F) included in the stud Each column represents one five year group

cushions Fifty arteries were counted in each section if a sufficient number of arteries was present

According to the percentage of myocardial arteries affected the hearts were classified into two groups

1 Ten per cent or less of the arteries affected
2 More than 10 per cent of the arteries affected

Macroscopical examination Total heart weight was noted The heart preparations included the first 2–3 cm of aorta Myocardial necroses or scars larger than one cm in any plane of section were recorded as infarcts The main coronary arteries and their larger branches were opened longitudinally until a luminal diameter of about one mm

According to a subjective estimation of the degree of atherosclerotic stenosis in the coronary arteries the hearts were classified into four groups

1 No stenosis
2 Stenosis reducing the original lumen by 50 per cent or less in one or more of the three main arteries
3 Stenosis reducing the original lumen by more than 50 per cent in one or more of the main arteries up to complete occlusion in two of the three main arteries
4 In all of the three main arteries complete atherosclerotic occlusion of the lumen

No attempt was made to differentiate atherosclerotic plaques from organized coronary thromb

Statistical methods employed in analysing the results were the χ square method with Yates correction in 2 by 2 contingency tables the χ square test for trend (8) Student s t test and Fisher s exact probability test (8)

RESULTS

The cushion like lesions were observed in one or more of the myocardial arteries in the majority of cases The frequency of affected arteries was about the same in different parts of the same heart



Fig 2

Intramyocardial artery containing a large intimal cushion rich in elastic material (dark grey) The lesion tapers at both sides and the elastic material merges with the internal elastic membrane (arrows) Gomori s aldehyde fuchsin × 710

The morphology of the cushions has been described elsewhere (6) They were elongated spindle shaped protrusions extending along the longitudinal axis of the arteries Predilection sites were at and distal to bifurcations and branching sites of small arteries In cross sections they appeared as cushion like subendothelial deposits tapering at one or both sides and merging with the internal elastic membrane (Fig 2) Disruption and new formation of elastic fibres was observed within the cushions together with modified smooth muscle cells or uncharacteristic rounded cells In some deposits a few mononuclear blood cells were present lipid and rarely material stained as fibrin could be seen

The intimal cushions were not found in any of the nine cases younger than 40 years These cases (7 males and 2 females) are therefore not included in the following analysis

Age and Sex

In groups of increasing age increasing proportions of men had more than 10 per cent of the myocardial arteries affected by cushion like intimal lesions (Table 1) In women the same trend was seen but it did not attain statistical significance at the 5 per cent probability level (Table 2)

The trend was largely unchanged when cases with coronary atherosclerotic stenosis grade 4 were excluded

In men younger than 55 years of age the frequency of arteries

TABLE 1

The Association between Age and the Occurrence of Myocardial Arteries Affected by Cushion Like Lesions in Men

	Total no of cases	Age group 40–64 no	65–74 no	75 + no
None to 10 per cent of the arteries affected by intimal lesions	83	38 (79 %)	29 (67 %)	16 (52 %)
More than 10 per cent of the arteries affected by intimal lesions	39	10 (21 %)	14 (33 %)	15 (48 %)
Total	122	48 (100 %)	43 (100 %)	31 (100 %)

χ^2 for the trend that the number of cases with more than 10 per cent of the myocardial arteries affected by cushion like lesions increases by groups of increasing age = 6.57 $0.025 > P > 0.01$

affected by cushion like lesions did not exceed 10 per cent in any heart Except for one woman of 47 this was also true for women Within each age group the frequency of affected arteries was about the same in both sexes

Coronary Atherosclerotic Stenosis

Cushion like intimal lesions in the myocardial arteries were less frequent in cases with pronounced coronary atherosclerotic stenosis than in cases with no or slight stenosis (Tables 3 and 4) This inverse relationship between lesions of the small and large arteries was statistically significant in both sexes

TABLE 2

The Association between Age and the Occurrence of Myocardial Arteries Affected by Cushion Like Lesions in Women

	Total no of cases	Age group 40–64 no	65–74 no	75 + no
None to 10 per cent of the arteries affected by intimal lesions	52	15 (68 %)	18 (72 %)	19 (51 %)
More than 10 per cent of the arteries affected by intimal lesions	32	7 (32 %)	7 (28 %)	18 (49 %)
Total	84	22 (100 %)	25 (100 %)	37 (100 %)

χ^2 for the trend that the number of cases with more than 10 per cent of the myocardial arteries affected by cushion like lesions increases by groups of increasing age = 2.10 $0.20 > P > 0.10$ NS

TABLE 3

The Association between Coronary Atherosclerotic Stenosis and the Occurrence of Myocardial Arteries Affected by Cushion-Like Lesions in Men

	Total no. of cases	Degree of coronary atherosclerotic stenosis			
		1	2	3	4
		no.	no.	no.	no.
None to 10 per cent of the arteries affected by intimal lesions	83	10 (50 %)	27 (61 %)	21 (70 %)	25 (89 %)
More than 10 per cent of the arteries affected by intimal lesions	39	10 (50 %)	17 (39 %)	9 (30 %)	3 (11 %)
Total	122	20 (100 %)	44 (100 %)	30 (100 %)	28 (100 %)
Median age		65.5	69	65	66.5

χ^2 for the trend that the number of cases with more than 10 per cent of the myocardial arteries affected by cushion-like lesions decreases by increasing degrees of coronary atherosclerotic stenosis = 9.44. $P < 0.005$.

TABLE 4

The Association between Coronary Atherosclerotic Stenosis and the Occurrence of Myocardial Arteries Affected by Cushion-Like Lesions in Women

	Total no. of cases	Degree of coronary atherosclerotic stenosis			
		1	2	3	4
		no.	no.	no.	no.
None to 10 per cent of the arteries affected by intimal lesions	52	15 (62 %)	11 (47 %)	7 (47 %)	19 (91 %)
More than 10 per cent of the arteries affected by intimal lesion	32	9 (38 %)	13 (53 %)	8 (53 %)	2 (9 %)
Total	84	24 (100 %)	24 (100 %)	15 (100 %)	21 (100 %)
Median age		63	74.5	73	74

χ^2 for the trend that the number of cases with more than 10 per cent of the myocardial arteries affected by cushion-like lesions decreases by increasing degrees of coronary atherosclerotic stenosis = 4.53. $0.05 > P > 0.02$.

There was no definite change in the degree of atherosclerotic stenosis among men within the age groups studied (Table 5). As regards women, those in the older age groups had more severe atherosclerotic stenosis than those in the younger (Table 6).

Heart Weight

No correlation was found between the prevalence of cushion like lesions and increasing heart weight in either sex.

No significant correlation of heart weight to age was observed. Exclusion of cases with diseases associated with increased or decreased heart weight[1] did not change this. This was also true when relative heart weight (*i.e.* total heart weight related to body height or body weight) was considered.

In both sexes the largest mean heart weight was observed in the hearts with pronounced degrees of coronary atherosclerotic stenosis.

TABLE 5

The Association between Age and Coronary Atherosclerotic Stenosis in Men

Degree of coronary athero sclerotic stenosis	Total no of cases	Age group 40 64	65–74	75 +
		no	no	no
1–2	64	24 (50 %)	21 (49 %)	19 (61 %)
3–4	58	24 (50 %)	22 (51 %)	12 (39 %)
Total	122	48 (100 %)	43 (100 %)	31 (100 %)

TABLE 6

The Association between Age and Coronary Atherosclerotic Stenosis in Women

Degree of coronary athero sclerotic stenosis	Total no of cases	Age group 40 64	65–74	75 +
		no	no	no
1–2	48	20 (91 %)	11 (44 %)	17 (46 %)
3–4	36	2 (9 %)	14 (56 %)	20 (54 %)
Total	84	22 (100 %)	25 (100 %)	37 (100 %)

χ^2 for association between age and degree of coronary atherosclerotic stenosis = 13.8, df = 2, $P < 0.01$

[1] *i.e.* 8 men and 53 women each with one or more of the following diseases: Old or healing myocardial infarct, myocardial fibrosis and heart failure, valvular heart disease, chronic alcoholism with or without liver cirrhosis, arterial hypertension, chronic renal disease, cerebral or subarachnoidal haemorrhage, ischaemic cerebrovascular disease, chronic obstructive lung disease and malignant neoplasm

TABLE 7
The Association between Heart Weight and Degree of Coronary Atherosclerotic Stenosis

Degree of coronary atherosclerotic stenosis	No of cases	Mean heart weight (gm)	± SD
Men			
1–2	64	428	130
3–4	58	511	160
Women			
1–2	48	366	121
3–4	36	438	137

t for difference in mean heart weight in men = 3.89 df = 120 $P < 0.001$

t for difference in mean heart weight in women = 2.71 df = 82 $P < 0.01$

(Table 7). When cases with diseases associated with increased or decreased heart weight were excluded, this trend was still seen, although not quite as distinct, and it did not attain statistical significance.

Certain Diseases

In the group of patients with *myocardial infarcts* of recent origin only (*i.e.* < 4 weeks) there was a low frequency of cases with more than 10 per cent of the myocardial arteries affected by cushion-like lesions compared with the remaining of the total material of 206 cases (Table 8). The same low prevalence of intimal cushions was found in the group of cases with both recent and old infarcts, although it is not statistically significant at the five per cent probability level.

In the group of patients with only old infarcts, the prevalence of cases with more than 10 per cent of the myocardial arteries affected by intimal cushions was about the same as in the remaining total material. The difference between the low prevalence among cases with only recent infarcts and the higher prevalence among cases with only old infarcts attains statistical significance ($P = 0.02$).

When men and women were regarded separately, the results in each sex did not differ from those presented above.

In *diabetes mellitus* the prevalence of cases with more than 10 per cent of affected myocardial arteries was low, but when compared with the prevalence of lesions in the rest of the entire series, the difference does not attain the level of five per cent probability (Table 8).

In *senile dementia* 6 out of the 7 cases had more than 10 per cent of affected myocardial arteries (Table 8).

In *cirrhosis of the liver* the corresponding numbers were 5 of 7 cases (Table 8).

TABLE 8

The Association between Certain Diseases and the Occurrence of Myocardial Arteries Affected by Cushion Like Lesions

Disease	No of cases	More than 10 % of the arteries affected No of cases	P for difference from total material §	Athero sclerotic stenosis grade 4 No of cases	Median age
Recent myoc infar t	23	2	<0 05	9	67 5
Recent and old myoc infarct	20	2	NS	14	71 5
Old myoc infarct	21	8	NS	5	67
Sudden coronary death	11	3	NS	7	72
Diabetes mellitus	14	1	NS	6	66 5
Malignant neoplasm	43	17	NS	4	67 5
Senile dementia	7	6	<0 005	2	83
Cirrhosis of liver	7	5	<0 05	0	73
Old rheum valvular dis	10	5	NS	5	78
Accidents acute intox	19	9	NS	0	72
Total material of cases 40 years of age and older	206	61		49	70 5

Some cases have been recorded more than once according to the presence of more than one of the listed diseases Men and Women are grouped together

§ All the P values represent the outcome of χ square tests or Fisher's exact probability test in 2 by 2 tables each disease group against the remaining total material

DISCUSSION

Intimal injury caused by haemodynamic mechanisms is supposed to be of major importance in the pathogenesis of the intimal cushion like lesions (6)

In the *present study the frequency* of intimal cushions appeared to increase with age and on the other hand severe coronary athero sclerotic stenosis was associated with a low frequency of intimal cush ions These findings do not conflict *with a theory of a haemodynamic* injury being involved in the pathogenesis of the cushions Firstly if the finding in old rats of a definite increase in susceptibility of coron ary arteries to induced intimal and medial lesions (11) also is valid for man this may imply that haemodynamic trauma is able to induce intimal lesions particularly in old age Secondly as severe arterial stenosis reduce distal blood flow and blood pressure (3 5 9) the haemodynamic mechanisms in the intramyocardial arteries may be modified by the development of severe stenosis in the main epicardial arteries This could be some of the reason for the sparcity of intimal cushions in cases with severe coronary stenosis

If haemodynamic trauma is of importance in the pathogenesis of the cushions a positive relation would possibly exist between the

cushions and high blood pressure. There was no correlation between the frequency of the cushion like lesions and increasing heart weight. However, in order to be able to draw any conclusions regarding heart weight and the occurrence of these lesions, it was also necessary to analyse (a) the relation of age and heart weight and (b) the relation of coronary atherosclerotic stenosis and heart weight. An inverse or positive correlation in (a) or (b) respectively would possibly obscure any connection between the frequency of cushion like lesions and heart weight.

(a) No significant correlation between age and heart weight was observed.

(b) A positive correlation between coronary atherosclerotic stenosis and heart weight was demonstrated. Therefore a possible correlation between the frequency of cushion like lesions and heart weight could have been obscured by the inverse correlation between lesions and severe coronary atherosclerotic stenosis.

Thus, provided that increased heart weight (more than 500 g in men and more than 450 g in women) reflects hypertension (2), there is no clear evidence that high blood pressure is related to a high frequency of cushion like lesions. On the other hand, such a relationship cannot be excluded.

Considering the various disease groups, it is possible that the low frequency of cushion like lesions in diabetes mellitus is partly related to the lower median age of subjects in this group, partly to the severe degree of atherosclerotic stenosis in the group.

In senile dementia, the high frequency of cushion like lesions may be explained by the old age alone.

In cirrhosis of the liver, severe coronary atherosclerotic stenosis was not present and the occurrence of many cushion like lesions is consistent with the general tendency of the lesions to be prevalent in cases with little or no stenosis in the large epicardial arteries.

The varying frequency of cases with the higher prevalence of cushion like lesions in the three infarct groups is somewhat difficult to understand on the basis of differences in age and atherosclerosis alone. In the group of patients with only old infarcts, the frequency of cases with coronary atherosclerotic stenosis grade 4 was somewhat lower than in the group with only recent infarcts. However, cases with the higher prevalence of cushion like lesions were much more frequent in the group of old infarcts compared with the group of recent infarcts. As the median age was about the same in the two groups, the possibility may be suggested that the passing through episodes of *myocardial infarcts* perhaps augments the intimal cushions.

Some augmenting effects of myocardial infarcts could possibly be due to factors causing endothelial injury. Such factors could be aggregates of erythrocytes seen in abnormal states (7, 12), increased reactivity of platelets in patients with recent or old myocardial infarcts

(1 10) or possibly the leucocytosis in recent infarction Vasospasm in the myocardial small vessels during infarction causing obstruction to the blood flow (4) could also possibly lead to endothelial injury

The low prevalence of cushion like lesions in the group of cases with both recent and old infarcts does not exclude the possibility of an augmenting effect of myocardial infarcts The frequency of severe atherosclerotic stenosis was high in this group and the general tendency of few cushions in cases with severe atherosclerotic stenosis may *counterbalance the possible augmenting effect* of an infarct

However these associations do not necessarily indicate a direct causal relationship

SUMMARY

In a previous work haemodynamic mechanisms were supposed to be of importance in the pathogenesis of cushion like thickenings in the intramyocardial arteries

In order to obtain further information about the pathogenesis of these lesions the frequency of the lesions in various conditions and diseases was studied in a consecutive autopsy series of 215 cases The lesions were found in the majority of cases older than 40 years and they were not seen in cases younger than this The prevalence of the lesions increased with age They were inversely related to severe coronary atherosclerotic stenosis The lesions were more frequent in cases with old myocardial infarcts than in those with recent infarcts

It is likely that haemodynamic mechanisms are of importance in the pathogenesis of the intimal cushions and the present results are consistent with this However other factors appearing in myocardial infarction may also be operating

REFERENCES

1 *Ardlie N G Kinlough R I & Schwart C J* In vitro thrombosis and platelet aggregation in myocardial infarction Brit med J *1* 888–891 1966
2 *Bell E T & Clawson B J* Primary (Essential) hypertension A study of 4?0 cases Arch Path *5* 939–1002 19?8
3 *Fiddian R V Byar D & Edwards F A* Factors affecting flow through a stenosed vessel Arch Surg *88* 83–89 1964
4 *Grayson J & Irvine M* Myocardial infarction in the monkey Studies on the collateral circulation after acute coronary occlusion Cardiovasc Res *2* 170–178 1968
5 *Gregg D E* Coronary Circulation in Health and Disease Lea & Febiger Philadelphia 1950 pp 180–183
6 *Hærem J W* Cushion like intimal lesions and platelet aggregates in intramyocardial arteries of man Acta path microbiol scand 77 73–89 1969
7 *Knisely M H Bloch E H Eliot T S & Warner L* Sludged blood Science *106* 431–440 1947
8 *Maxwell A E* Analysing Qualitative Data Third ed Methuen & Co Ltd London 19?7 pp 46–7?
9 *May A G De Weese J A & R b C G* Hemodynamic effects of arterial stenosis Surgery 53 513–524 1963

10 *O'Brien J R*, *Heywood J B* & *Heady J A* The quantitation of platelet aggregation induced by four compounds A study in relation to myocardial infarction Thromb Diath haem *16* 752-767 1966
11 *Patek P R*, *de Mignard V A* & *Bernick S* Changes in structure of coronary arteries Susceptibility to arteriosclerosis in old rats Arch Path *85* 388-396 1968
12 *Wells R*, *Herman M V* & *Gorlin R* Microvascular changes in coronary artery disease Circulation *34* Suppl *3* 237 1966

Acta path microbiol scand 77 609 616 1969

The University Institute for Experimental Medicine Nørre alle 71
2100 Copenhagen Ø

CHANGES IN SENSITIVITY TO ANGIOTENSIN AND RENIN

Studies on Normal Nephrectomized, Ureterligated Hypoxic Partially Corticectomized or Medullectomized and Renal or Spontaneously Hypertensive Rats

By

JENS BING

Received 19 v 69

It is wellknown that the sensitivity of rats to angiotensin and renin and other pressor substances is rather different even when normal rats of the same strain age weight and sex are used Such variation in the sensitivity is also found when nephrectomized rats are used for the as says but normal and nephrectomized rats differ in their pattern of response to renin the nephrectomized responding with an increased and prolonged blood pressure rise (for literature see *Page & McCubbin*, 1968) The varying results of studies on this change in sensitivity seem to be due to the fact that many investigators have used different species of experimental animals and different forms of anaesthetics pretreatment of the animals and of doses and purity of the renin pre parations

It has been the aim of the present study to obtain further information about the mechanism causing the changed sensitivity to renin in nephrectomized rats For this purpose sensitivity to angiotensin and renin were studied in normal nephrectomized ureterligated hypoxic partially corticectomized or medullectomized and renal or spontaneously hypertensive rats The reason for using these differently pretreated rats was partly that they were used in a previous study on experimentally induced changes in plasma angiotensinogen and plasma renin (*Bing & Poulsen* 1969) and partly that it was thought of interest to compare the changed sensitivity of nephrectomized rats with that of rats with other forms of changed renal morphology and (or) function The study of ureterligated rats should thus allow an estimate of the influence of uraemia the partially corticectomized rats and those partially medullectomized being thought to illustrate the influence of lack of cortical or medullary tissue the study of hypertensive rats could show the influence of the blood pressure level

---

Supported by a grant from *King Christian X s Foundation*

MATERIAL AND METHODS

Animals White female rats weighing 180-200 grams from the strain of Wistar from Leo Pharm Trade Co were used for nearly all experiments The rats with spontaneous hypertension belonged to the strain of S A from The Robert Jones and Agnes Hunt Orthopaedic Hospital England[1] The material includes 69 rats eighteen normal seven 22-24 hours nephrectomized eight 22-24 hours ureterligated ten 16-18 hours hypoxic five 22-24 hours partially corticectomized six 22-24 hours partially medullectomized nine renal hypertensive rats studied about 3 months after a clamp was placed on one of the renal arteries and six rats with spontaneous hypertension

The methods for partial *corticectomy* and *medullectomy* partial clamping of the renal artery and hypoxia were those recently described (*Bing & Poulsen* 1969)

The *pressor response* to angiotensin and renin was studied in rats anaesthetised with 20 mg of amytal and pretreated with 5 μg of ergotamine tartrate 1 2 and 2 4 ng of angiotensin research Standard A and 14 mU of Haas Goldblatt Standard (G U) Renin being injected intravenously

RESULTS

1 *The Maximum Increase in Blood Pressure*

In *normal* rats the pressor response to intravenous injection of 2 4 ng of angiotensin was found to vary from about 10 to about 40 mm Hg with a single value of well over 50 mm Hg and the pressor response to 14 mU of Haas Goldblatt renin to follow the variations in responsiveness to angiotensin so that the maximum rise in the individual rat was about the same the response to angiotensin being about 90 per cent of the response to renin (Fig 1) Normal *relation between sensitivity to angiotensin and renin* was also found in the spontaneously hypertensive rats but both in the operated and in the hypoxic rats the maximum increase in blood pressure was markedly higher after injection of renin than after injection of angiotensin (Fig 1 and 2) In renal hypertensive rats the response to angiotensin was somewhat higher than the response to renin The deviation from the normal ratio between the responses to angiotensin and renin was due to changes in both

The sensitivity to angiotensin was markedly decreased in all non normal groups While nearly all normal rats responded to 2 4 ng angiotensin with a more than 20 mm Hg high increase in blood pressure the increase was somewhat lower in *hypoxic* rats (about 10-20 mm Hg) still lower (about 7-20 mm Hg) in *ureterligated nephrectomized partially corticectomized* and *spontaneously hypertensive* rats and very low (5-10 mm Hg) in partially *medullectomized* and in *renal hypertensive* rats

The sensitivity to renin was changed in another way While most of the *nephrectomized* and partially *corticectomised* rats reacted to 14 mU of renin with pressor responses of 30 to well over 50 mm Hg thus being similar to or slightly higher than those found in most *normal* rats (Figs

---

[1] The author is thankfull to prof *Morten Simonsen* for supplying the S A rats

[2] Both standards were kindly given us from the division of biological standards Medical research council Mill Hill London

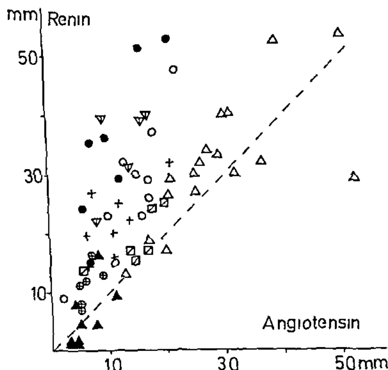


Fig 1

Relation between pressor response in mm Hg to 14 milli Units Haas Goldblatt renin and 2 4 ng of angiotensin in rats

△ normal	▽ partially corticectomized
● nephrectomized	⊕ medulle tomized
+ ureterligated	⊠ spontaneously hypertensive
○ hypoxic	▲ renal hypertensive

The dot—and—dash line includes identical responses to renin and angiotensin

1 2 and 3) the *hypoxic* rats reacted as the normal rats Contrary to these most *ureterligated* and *spontaneously hypertensive* rats reacted with a relatively low response to renin (16–30 mm Hg) the partially *medullectomized* rats with only 7–17 mm Hg and 9 *renal hypertensive* rats with a mean of 8 mm Hg (range 0–17) 6 of them responding with less than 6 mm Hg

2 *The Duration of the Pressor Response*

The typical short normal response to 2 4 ng of *angiotensin* was found in all groups (Figs 2 and 3) It was further found that cessation of continuous injection of 9 ng of angiotensin per min was followed by the same abrupt fall in blood pressure in nephrectomized and in normal rats

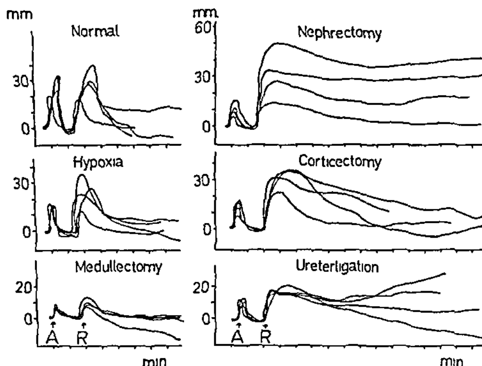


Fig 2

Representative curves showing the pressor response to 2 4 ng of angiotensin (A) and 14 mU of renin (R) in normal 22–24 hours nephrectomized ureterligated partially corticectomized or medullectomized and 17 hours hypoxic rats

The duration of the pressor response and the form of the curve after injection of 14 mG U *renin* are seen in Figs 2 and 3 Normal rats respond to renin with a rise the velocity of which in most cases is a little slower than that seen after *injection of angiotensin followed by a still* slower return of the pressure to the starting level the duration of the whole response in most cases being about 3–6 minutes While this level is reached in most cases the response can in some cases one of which is shown in Fig 2 stop at a higher level This is most often found when the blood pressure at the time of the injections is lower than that found immediately after the start of the experiment While the duration of the response to renin is normal in hypoxic and spontaneously hypertensive and often shorter than normal *in partially medullectomized and renal* hypertensive rats it is prolonged lasting for more than 30 60 minutes in nephrectomized and ureterligated rats being somewhat less prolonged in partially corticectomized rats (Fig 2)

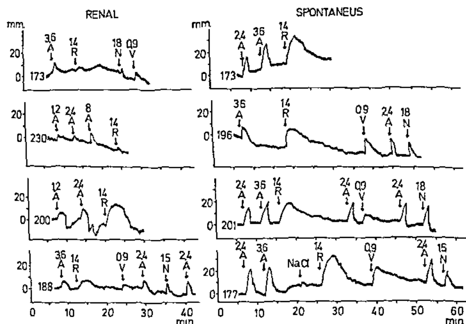


Fig 3

Pressor responses to angiotensin (A) renin (R) vasopressin (V) and noradrenaline (N) in 4 renal hypertensive rats and in 4 rats with spontaneous hypertension The values given are ng for angiotensin in Haas Goldblatt Units for renin mU for vasopressin and ng for noradrenaline *The value given in front of each curve in dicates the blood pressure in mm Hg*

DISCUSSION

1 *Cause of the Sometimes Increased and Always Markedly Prolonged Pressor Response to Renin in Nephrectomized (and Partially Cor ticectomized) Rats*

In most studies on the abnormal response to angiotensin and renin in nephrectomized animals the response to angiotensin has been found to be normal or increased and the response to renin changed being both increased and prolonged The present investigation is so far in agree ment with these results as the mean response to renin is higher in the nephrectomized than in the normal rats and the prolonged type of the curve (Fig 2) is quite the same as that previously found But the max imum increase in response to renin in nephrectomized rats is markedly smaller than that reported in the literature and the decrease in response to angiotensin has apparently not been observed in previous studies The reason for these differences is probably to be found in differences in animals anaesthesia pretreatment and renin preparations and doses used in studies like the present

The mechanism involved in the changed response to renin in ne phrectomized animals is unknown Many of the primarily possible cau

ses seem to be excluded (see *Page & McCubbin* 1968) Some of the remaining possibilities will be discussed here

1 The changed response to renin in nephrectomized animals seems not to be due to their increased *plasma angiotensinogen*, as *Carretero & Gross* (1967 b) found the changed response but only slightly increased angiotensinogen in rats tested two hours after nephrectomy The present finding of normal or shortened duration of the renin pressor curves in hypoxic and in partially medullectomized rats in defiance of their markedly increased plasma angiotensinogen (*Bing & Poulsen* 1969) is in agreement with this conclusion

2 According to recent studies by *Sen et al* (1968) the kidneys contain a *phospholipid* which both reduces the blood pressure of renal hypertensive rats and inhibits the pressor response to renin in normal as well as in nephrectomized rats This finding opens up the possibility that removal of this renal phospholipid by nephrectomy is the cause of the changed pressor response The location of the factor inside the kidney has not been determined but other vasodepressive renal factors have been shown to be located to the medulla The present finding of about the same changes in pressor response to renin in partially corticectomized and nephrectomized rats and exactly the opposite changes in partially medullectomized rats shows that if lack of this factor should be the cause of the changed response it must be located to the cortex

3 *Bunag et al* (1968) found that the enhancement of the maximum pressor response in nephrectomized dogs was inhibited if they were pretreated with crude kidney extract hog renin or angiotensin and they therefore believe that the increased pressor response is due to the *depletion of endogenous renin* caused by the nephrectomy Their study does not allow any evaluation of the influence of the treatment with renin or angiotensin on the prolongation of the pressor response which is found after nephrectomy As ureterligated rats have been found to have much higher plasma renin than nephrectomized (and corticectomized) rats (*Bing & Poulsen* 1969) the present finding that the prolongation of the pressor response to renin is quite similar in ureterligated and in nephrectomized rats speaks against the hypothesis that the prolonged response to renin is due to depletion of endogenous renin

4 In a previous study it was found that *uraemia* might perhaps be the cause of the increased angiotensinogen concentration in nephrectomized and ureterligated rats (*Bing & Poulsen* 1969) The present finding about the same prolonged response to renin in nephrectomized partially corticectomized and ureterligated rats could speak for a causal relationship between uraemia and prolonged response to renin The finding of just the same degree of uraemia in partially medullectomized and in partially corticectomized rats both of which have plasma creatinine concentrations of 3 to 4 mg per cent however speaks against this

explanation as the medullectomized rats do not react with a prolonged but on the contrary with a shortened response to renin

5 The typical prolongation of the response to renin in nephrectomized animals cannot be explained by a prolonged persistence of the injected renin in the blood of nephrectomized animals as *Schaechtelin et al* (1964) found the same half life for renin in plasma of normal and nephrectomized rats They therefore assumed that an *accumulation of renin in the arterioles* might be responsible for the prolonged response This hypothesis was supported in studies showing that anti angiotensin injected in doses which neutralizes renin in normal rats and angiotensin both in normal and nephrectomized rats will only diminish the maximum increase and will not change the prolonged form of the response to renin in nephrectomized rats *(Bing & Poulsen* (1968) A direct proof of the correctness of this hypothesis by determinations of the arteriolar renin content after renin injection into normal and nephrectomized animals however is lacking

2 *Cause(s) of the Low but Markedly Prolonged Response to Renin in Ureterligated Rats*

The cause(s) of the changed response to angiotensin and renin in ureterligated rats (Fig 2) is (are) still unknown It is possible that the decrease in maximum response is caused by the same unknown mechanism which causes the still lower response in partially medullectomized and renal hypertensive rats The cause of the prolongation of the response to renin may be the same as that which causes the prolonged response in nephrectomized and partially corticectomized rats

3 *Cause(s) of the Low response to Angiotensin and Low and Short Response to Renin in Partially Medullectomized and Renal Hypertensive Rats*

The cause(s) of the changed response to angiotensin and renin in partially medullectomized and renal hypertensive rats (Figs 2 and 3) is (are) unknown The present study shows that the change in response to renin so far should not be believed to be due to an inhibitor of the reaction between renin and its substrate angiotensinogen as the response to angiotensin is equally or still more reduced The very low response to angiotensin and renin in renal hypertensive rats seems not to be due to the high level of the blood pressure as spontaneously hypertensive rats with the same blood pressure level have only moderately reduced response (Fig 3) Nor can it be due to angiotensin—and renin tachyphylaxis—as the responses to noradrenaline and vasopressin were similarly reduced (Fig 3)

SUMMARY

1 The *sensitivity to angiotensin* was found to be somewhat decreased in hypoxic rats more decreased in ureterligated nephrectomized partially corticectomized and spontaneously hypertensive rats and very low in partially medullectomized and in renal hypertensive rats

2 The *sensitivity to renin* was as regards the *maximum increase* normal or increased in nephrectomized and partially corticectomized rats normal in hypoxic rats somewhat decreased in ureterligated and spontaneously hypertensive rats markedly decreased in partially medullectomized rats and still more decreased in renal hypertensive rats in which the sensitivity to angiotensin noradrenaline and vasopressin was about equally low The *duration* of the pressor response was about equally markedly prolonged in nephrectomized and ureter ligated rats and also markedly but somewhat less prolonged in partially corticectomized rats

3 The most recent hypotheses dealing with the explanation of the *mechanism of the changed response to renin in nephrectomized rats* are discussed in relation to the results of the present study It is concluded that the prolonged response to renin in nephrectomized rats is not due either to increased plasma angiotensinogen or a medullary inhibitor of the renin angiotensinogen reaction nor to depletion of endogenous renin or to uraemia

4 The mechanism of the *changed response to angiotensin and renin* (decreased maximum response to both and prolonged response to renin) in *ureterligated* rats is unknown The decreased response to both angiotensin and renin in *renal hypertensive rats* and in *partially medullectomized* rats seems not to be due to an inhibitor of the reaction between renin and angiotensinogen nor to the blood pressure level or to renintachyphylaxis

REFERENCES

Bing J & Poulsen K In vivo effects of anti angiotensin II Acta path microbiol scand 74 139–140 1968

Bing J & Poulsen K Experimentally induced Changes in Plasma Angiotensinogen and Plasma Renin Acta path microbiol scand 77 389–398 1969

Bunag R D McCubbin J W & Page I H Increased Cardiovascular Reactivity Caused by Depletion of Endogenous Renin in Dogs Circ Res 23 709–716 1968

Carretero O & Gross F Evidence for humoral factors participating in the renin substrate reaction Circ Res 20/21 *Suppl II* 115–126 1967

Page I H & McCubbin J W Renal Hypertension Chicago 1968 page 30

Schaechtelin G Regoli D & Gross F Am J Phys 206 1361–1364 1964

Sen S Semby R R & Bumpus F M Antihypertensive effect of an isolated phospholipid Am J Phys 214 337–341 1968

Aoki K & Mason G M C Recently found decreased sensitivity to renin and angiotensin in unanesthetized renal hypertensive rats with one clipped and one untouched kidney but normal response in rats with a sole clipped kidney Nephron 6 484–497 1969

Acta path microbiol scand 77 617-6?? 1969

The Department of Oral Pathology Royal Dental School Copenhagen Denmark

HISTORADIOGRAPHIC STUDY OF EXFOLIATED CELLS OF THE ORAL MUCOSA

A Method for Comparison of Historadiograms and Papanicolaou Staining

By

E DABELSTEEN and F P CLAUSEN

Received 25 vi 69

Investigations of the degree of keratinization of exfoliated cells of the oral mucosa have mainly been based on smears stained according to the method described by Papanicolaou (1942) Most of these investigations have been reviewed by *Camilleri & Lange* (1966)

Although the reliability and usefulness of the smear technique as a means of detecting malignances and premalignances has been questioned among others by *Gorlin* (1965) and *Chandler* (1966) the value of the Papanicolaou staining technique for estimating degree of keratinization of squamous epithelial cells is generally trusted This confidence is reflected by the great amount of published studies based on this method

A few authors however *Silverman Becks & Farber* (1958) *Trott* (1962) *Trott & Banoczy* (1962) and *Waller* (1962) have expressed their doubt as to the value of the Papanicolaou staining for evaluating the degree of keratinization of exfoliated cells

An incontestable disadvantage of the Papanicolaou method is its unsuitability for quantitative studies *Onisi & Kesuge* (1963) overcame this difficulty by using the crystal violet and Bismarck brown of a Gram stain Dyes in specimens each containing numerous cells were dissolved in acetone and analyzed by means of an electrophotometer This method however does not allow an estimation of the degree of keratinization of the single cell and the factors influencing the staining reactions are not well understood

Historadiographic studies of skin and oral mucous membrane have shown a very considerable difference in X ray absorption—and hence in dry weight—between the keratinized and non keratinized cell layers of the squamous cell epithelium *Moberger & Engstrom* (1954) *Lindstrom & Moberger* (1955) and *Clausen* (1969) *Muller Sandritter &*

Supported in part by a grant DE 1358 from the *National Institute of Dental Research National Institutes of Health U S P H S Bethesda Maryland*

Schwaiger (1959) developed a method for historadiographic determination of dry weight without use of a reference system using a sealed-off X-ray tube (*Combée, Houtman & Recourt* (1955). *Müller et al* (1959) showed that this method could be used for quantitative determination of the dry weight of exfoliated squamous epithelial cells of the oral mucous membranes with a mean error of reproducibility of about 10 per cent.

The usefulness of the historadiographic method of studying exfoliated cells and the conspicuous difference in dry weight between keratinized and non-keratinized cell layers made the authors believe that the historadiographic method could be used to study exfoliated cells of the oral mucosa from patients with disturbances of keratinization. It was also believed that the method could be used to estimate the reliability of the Papanicolaou technique as an indicator of degree of keratinization.

Therefore the purpose of the present investigation has been to work out a method which allows a historadiographic examination and a Papanicolaou staining as well of the same exfoliated cell.

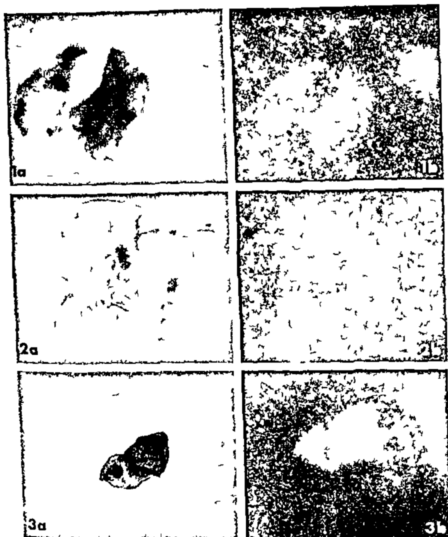
MATERIAL AND METHOD

The studied material consisted of exfoliated cells from the oral mucosa from clinically normal cheeks and hard palates of adult men. The scrapings were made with a wooden cotton covered stick moistened in physiologic saline solution. They were taken from the area just anterior to the papilla salivalis superior and from the hard palate. In order to obtain unkeratinized and keratinized cells on the same historadiogram cells from cheek and palate of the same person in some cases were mixed in the same specimen. The sticks were immediately rolled directly onto the emulsion side of an Agfa Gevaert 10E56 film and fixed for 30 minutes in a solution of 96 per cent alcohol and were then air dried.

The films were exposed in a Philips CMR 5 contact microradiograph with equipment very similar to that used by *Müller et al* (1959). For a detailed description of the equipment see *Clausen & Dahlin* (1969). The exposures were made for 40 minutes in continuously evacuated exposure chamber. The voltage across the X-ray tube was 1.5 kV which with the equipment used gives a radiation of the specimen with polychromatic X-rays ranging from a wave length of 8.2 Å to 13 Å. The maximum intensity was about 12.5 Å. After exposure the film was developed for 5 minutes in Gevaert's developer GP 201 diluted 1:4 with distilled water and then fixed and rinsed thoroughly. Thereafter the film with exfoliated cells was stained according to Papanicolaou's technique (*Clayden* 1962). For the purpose of identification of the cells and for comparison of the colour and the radiopacity of the cells all the radiograms were mounted in water between slide and cover glass and microphotographed on Agfa CK 20 colour film. Under a drop of water and observed through the low power of a microscope the cells were then removed one by one from the film by means of a nylon hair and the historadiograms were dried and mounted in Eukitt® between histologic slides and cover glasses the emulsion side facing the cover glass. The historadiograms were examined under a microscope and compared with the colourslides of the corresponding cells.

RESULTS

The method is circumstantial and time consuming. Although it implies many possibilities of making artifacts it was successful in nearly all cases. Only in 5 instances was the photographic emulsion damaged



Figs 1–3

Fig 1 a + b The lowest radiopacity is seen in the blue cell there is a higher radiopacity in the red cell and the highest radiopacity is seen in the yellow cell This seems to indicate an increasing dry weight with increasing degree of keratinization and a good accordance between the latter and the staining reaction Note in the cell to the left that the nucleus appears more radiopaque than the surrounding cytoplasm × 300

Fig 2 a + b A faintly and a strongly stained blue cell Note that the strongly stained cell shows a considerably higher radiopacity than the faintly stained cell of the same colour × 300

Fig 3 a + b Two small cells with pyknotic nuclei Both show a high radiopacity of nearly equal intensity in spite of an entirely different staining reaction Note the shrinkage of the cells (a) as it results from the staining it does not influence the radiogram × 300 The relation between colour and radiopacity shown in Fig 2 and 3 possibly indicates a disagreement between staining reaction and degree of keratinization

during the procedure The Papanicolaou stained cells were categorized according to the colour of the cytoplasm into yellow (keratinized) red (partially keratinized) and blue (non keratinized) types

Examination of about 17 stained scrapings and the corresponding historadiograms showed that blue cells generally had a less radio paque cytoplasm than the red cells (Fig 1 A + B) However besides this correlation between the radiopacity and the quality of the colour of the cell there seemed to be a correlation between the intensity of the colour of the cell and the degree of its radiopacity in such a way that heavily stained blue red or yellow cells or parts of these were more radiopaque than lightly stained areas (Fig 2 A + B) Although these were the most common findings cells could be found with different colours and with a very similar radiopacity (Fig 3 A + B) The nuclei of the cells were more radiopaque than even the most radiopaque areas of the surrounding cytoplasm independent on the colour of the cyto plasm (*Fig 1 A + B*)

DISCUSSION

As shown by *Engstrom & Lindstrom* (1949 1950) and by *Lindstrom* (1955) ultrasoft X rays can be used for the determination of the dry weight of cells in de paraffinized sections and in frozen dried sections of soft tissue provided that they are basically built up of carbon oxygen and nitrogen In the wave length region used 8–13 Å the mass absorption coefficients of high atomic elements are greatly reduced and in oral mucosa high atomic elements are present in very small concen trations as compared with the carbon oxygen and nitrogen of the proteins Only sulphur might be suspected to give a stronger X ray absorption than to its mass According to *Moberger & Engstrom* (1954) it can be calculated however that even so high a sulphur concentra tion as 10 per cent does not disturb the dry weight determination Furthermore recent studies quoted by *Silverman* (1967) confirm that epidermal keratin differs from some other animal keratins in that it is a very low sulphur containing protein The relative radiopacity of the cell structures in historadiograms hence reflects the relative distri bution of dry weight in the cells

The technique introduced by *Muller et al* (1959) made it possible to use the relative simple Philips CMR 5 microradiograph for quantita tive studies without a reference system and demonstrated the pos sibility of determining the dry weight of among other things exfoliated cells of the oral mucosa The present authors have improved the tech nique further by applying the cells directly onto the emulsion of the film thus avoiding the 7 µm thick Mylar film formerly used which has a much too high absorption a drawback of which *Muller et al* (1959) (p 430) were very well aware The direct application gives *the histo* radiograms a far better sharpness

As the purpose of the present study was to work out a method to compare the historadiogram and the Papanicolaou staining of the very same cell it was necessary to stain the cells while they were still on the radiogram if identification of cells should not be made unreasonably difficult In this way a faint staining of the emulsion cannot be avoided It is important that the Papanicolaou staining is performed after exposure development and fixation of the historadiogram in order to avoid the influence of absorption of X rays by the dyes

The present method offers considerable advantages compared with the quantitative estimation of the staining of exfoliated cells made by *Onisi & Kosuge* (1963) the historadiographic technique being easier to quantitate and reproduce than the staining technique and because the estimation can be done on the single cell

Most authors agree that differences in colours of the Papanicolaou stained cells reflect differences in keratinization If this assumption is correct one should expect blue cells to have a low radiopacity and hence a low dry weight The radiopacity is supposed to increase in red and yellow cells In most cases this is what has actually been found Nevertheless in quite a few cases the degree of radiopacity seemed to be related to the intensity of the staining and not to the colour per se This correlation might be caused by differences in thicknesses of the cells or it might indicate that the Papanicolaou technique is unreliable as a method of estimating degree of keratinization of exfoliated cells of the oral cavity To find out which is the case it will be necessary to make cytophotometric measurements of the historadiograms and the stained cells and measurements of the thickness of the cells and then correlate the findings

On historadiograms of human oral mucous membranes (*Moberger & Engstrom* 1954 *Clausen* 1969) the nuclei of the epithelial cells present themselves as relatively radiolucent areas with exception of the nuclei of the parakeratotic layer when they consist of intensely compressed cells On the historadiograms of the exfoliated cells the nuclei when present are invariably radiopaque This might be explained by the fact that although the nuclei have a lower dry weight than the surrounding cytoplasm and will thus appear relatively radiolucent on tissue section they will appear relatively radiopaque in the exfoliated cells because of the fried egg form of these cells In spite of the relatively lower absorption of the nucleoproteins compared with that of the plasmaproteins the differences in thickness of the nucleus and the cytoplasm make the former appear the more radiopaque

SUMMARY

A method is presented which allows the examination of historadiograms and Papanicolaou staining of the same exfoliated cells of the oral cavity The historadiograms have been made in a Philips CMR 5

contact microradiograph under standardized conditions The radiation consisted of polychromatic X rays ranging in wave length from 8 2 Å to 13 Å The exfoliated cells were placed directly onto the photographic emulsion and the Papanicolaou staining was performed while the cells were still on the film Examination of the historadiograms and the stained cells suggested that the Papanicolaou technique is possibly not a completely reliable method of estimating exactly the degree of keratinization of exfoliated cells This problem however can only be solved on the basis of a quantitative photometric examination of the historadiograms prepared by means of the technique described comparing the results with the thicknesses and staining reactions of the corresponding cells

REFERENCES

Camilleri G E & Lange D Exfoliative cytology—a review of its application to non neoplastic conditions Int dent J *16* 311–327 1966

Chandler J R The nonvalue of oral cytology Arch Otolaryng *84* 527–533 1966

Clausen F P Studies in oral leukoplakias Historadiographic study of oral leukoplakias Manuscript 1969

Clausen F P & Dahlin J Application of ultra soft X ray historadiography in the study of oral soft tissues Manuscript 1969

Clayden E C Practical section cutting and staining 4 ed J & A Churchill Ltd., London 1962 p 134–135

Combee B Houtman J & Recourt A A sealed off X ray tube for contact micro radiography Brit J Radiol 28 537–542 1955

Engstrom A & Lindstrom B A new method for determining the weight of cellular structures Nature *163* 563 564 1949

Engström A & Lindstrom B A method for determination of the mass of extremely small biological objects Biochim biophys Acta *4* 351–373 1950

Gorlin R J The limitations of exfoliative cytology in oral cancer detection CA (N Y) *15* 82–84 1965

Lindstrom B Roentgen absorption spectrophotometry in quantitative cytochemistry Acta radiol (Stockh) Suppl *125* 206 pp 1955

Lindstrom B & Moberger G The distribution of the dry weight in squamous epithelium during carcinogenesis studied by means of quantitative roentgen absorption spectrophotometry Exp Cell Res *9* 68–87 1955

Moberger G & Engstrom A Historadiographic studies on normal hyperplastic and cancerous epidermis J invest Derm *22* 477–491 1954

Muller D Sandritter W & Schwaiger C Eine Methode zur Rontgenhistoradiographischen Trockengewichtsbestimmung ohne Verwendung eines Referenz systems Histochemie *1* 420–437 1959

Müller D Sandritter W Schiemer H G & Endres K Rontgenhistoradiographische und interferenzmikroskopische Trochengewichtsuntersuchungen an Zellausstrichen Histochemie *1* 438–444 1959

Onisi M & Kosuge H Keratinization of the oral mucosa J dent Res *42* 757 1963

Papanicolaou G N A new procedure for staining vaginal smears Science *95* 432 1942

Silverman S Jr Ultrastructure studies of oral mucosa I Comparison of normal and hyperkeratotic human buccal epithelium J dent Res *46* 1433–1443 1967

Silverman S Jr Becks H & Farber S M The diagnostic value of intraoral cytology J dent Res *37* 195–205 1958

Trott J R A desquamative cytological study of healthy oral mucosa Acta anat *49* 289–296 1962

Trott J R & Bandoczy J The oral mucosa and keratinization Acta morph Acad Sci hung *11* 217–227 1962

Walker R O The oral mucosa as an index of health Brit J clin Pract *16* 699–704 1962

Acta path microbiol scand 77 6?3-638 1969

The Gade Institute Department of Pathology University of Bergen Norway
(Head Prof F Waaler M D)

ORAL POLYETHYLENE GLYCOL'S INHIBITORY EFFECT ON THE SUBCUTANEOUS GROWTH OF EHRLICH'S CARCINOMA IN MICE, AND ON THE LOCAL INFLAMMATORY RESPONSE TO THE TUMOUR

By

F Hartveit

Received 21 vi 69

Polyethylene glycol is a long chain polymer which can potentiate the haemolytic (*McVicar* 1949) and the oncolytic (*Hartveit* 1967) activity of complement It has previously been reported (*Hartveit* 1969) that intraperitoneal treatment with polyethylene glycol 4000 has an inhibitory effect on the subtaneous growth of Ehrlich s carcinoma in female mice of our closed colony It was subsequently remarked that a 20 per cent solution of polyethylene glycol 4000 administered *ad lib* in the drinking water had a similar inhibitory effect In a preliminary experiment 12 female mice given this treatment showed a 48 per cent reduction in subtaneous tumour growth 9 days after transplantation compared to 12 untreated controls (*0 001 > P*)

The mechanism underlying this growth inhibition has not been studied previously The experiment was therefore repeated (Exp 1) and extended to include male mice (Exp 2) and histological studies Oral treatment with polyethylene glycol 4000 was accompanied by profuse watery diarrhoea and marked weight loss A further control study was therefore included (Exp 3) in which the mice were given glycerol per os in an attempt to evaluate the part played by dehydration per se

In the course of the experiments it became apparent that the inflammatory response to the tumour was a determining factor in its growth This is in keeping with Jones observation (1914) that the presence of a non specific irritant may enhance tumour growth and *Wheatley* s (1963) comparable findings with ascitic growth of this tumour The importance of new connective tissue proliferation has been stressed

---

Requests f r reprints should be addressed to Dr F Hartveit Gades Institutt Haukeland Sykehus 5000 Bergen Norway

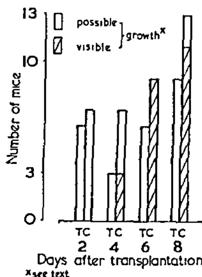


Fig 2
Tumour growth in mice treated with polyethylene glycol (T) and in untreated controls (C) related to days after transplantation

Spleen Weight

At 8 days after transplantation the mean spleen weight ± S D in the polyethylene glycol treated mice was 44 2 ± 25 6 mg while that the controls was 145 3 ± 62 4 mg This difference is statistically significant *(0 001 > P)*

There was no significant correlation between tumour size and final body weight or between body weight and spleen weight at 8 days in either group

Experiment 2 (20 per cent and 10 per cent polyethylene glycol in male mice) The *body weight* of these male mice is also shown in Fig 1 The standard deviation from the means was between 1 4 and 2 9 g The weight of the mice treated with 20 per cent polyethylene glycol fell steadily at about the same rate as that of the females in experiment 1 the weight loss at 8 days being 26 per cent Treatment with 10 per cent polyethylene glycol gave little change in weight which remained similar to that in the controls The difference in weight at 8 days in the mice treated with 20 per cent polyethylene glycol and the un treated controls is statistically significant *(0 001 > P)*

Tumour growth following 20 per cent polyethylene glycol was similar to that recorded in the females in Exp 1 By 8 days possible growth was present in only 4 of the treated mice while growth was visible in 9 and possible in 3 of the untreated controls Following a 10 per cent solution of polyethylene glycol growth was similar to that in the controls

It is of note that the amount of polyethylene glycol taken by the

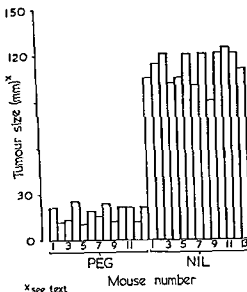


Fig 3
Tumour size at 8 days in mice treated with polyethylene glycol (PEG) compared to that in untreated controls (NIL)

mice given the 10 per cent solution was probably much less than half that taken by those given the 20 per cent solution as the bottles for the latter had to be filled twice as often as the others

Tumour si e as judged from the sum of the two diameters at 8 days was significantly reduced in the mice treated with 20 per cent polyethylene glycol compared to that in the controls (29 1 ± 5 2 mm and 36 7 ± 6 0 mm resp *0 01 > P > 0 001*) Following 10 per cent polyethylene glycol growth was similar to that in the controls The tumour depth varied from 1–2 mm in those given 20 per cent polyethylene glycol and from 2–4 mm in the others Judged from the sum of the diameters multiplied by the depth the group given 20 per cent polyethylene glycol showed a mean reduction in tumour growth of 48 per cent compared to the untreated controls

Spleen weight Following 20 per cent polyethylene glycol the mean weight ± S D was 59 2 ± 28 6 mg following 10 per cent the weight was 151 3 ± 74 0 mg and 153 2 ± 69 8 mg in the controls The difference in spleen weight between those given 20 per cent polyethylene glycol and the untreated controls is statistically significant (*0 001 > P*)

The relationships between final body weight and spleen weight and between tumour size and body weight were also investigated There was no significant correlation between any of these factors except for the tumour size and body weight in the group treated with 20 per cent polyethylene glycol where the positive correlation was just statistically

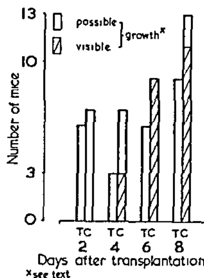


Fig 2

Tumour growth in mice treated with polyethylene glycol (T) and in untreated controls (C) related to days after transplantation

Spleen Weight

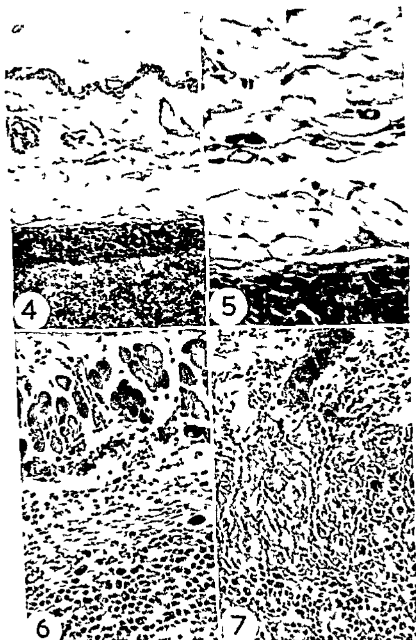
At 8 days after transplantation the mean spleen weight ± S D in the polyethylene glycol treated mice was 44 2 ± 25 6 mg while that the controls was 145 3 ± 62 4 mg This difference is statistically significant ($0.001 > P$)

There was no significant correlation between tumour size and final body weight or between body weight and spleen weight at 8 days in either group

Experiment 2 (20 per cent and 10 per cent polyethylene glycol in male mice) The *body weight* of these male mice is also shown in Fig 1 The standard deviation from the means was between 1 4 and 2 9 g The weight of the mice treated with 20 per cent polyethylene glycol fell steadily at about the same rate as that of the females in experiment 1 the weight loss at 8 days being 26 per cent Treatment with 10 per cent polyethylene glycol gave little change in weight which remained similar to that in the controls The difference in weight at 8 days in the mice treated with 20 per cent polyethylene glycol and the untreated controls is statistically significant ($0.001 > P$)

Tumour growth following 20 per cent polyethylene glycol was similar to that recorded in the females in Exp 1 By 8 days possible growth was present in only 4 of the treated mice while growth was visible in 9 and possible in 3 of the untreated controls Following a 10 per cent solution of polyethylene glycol growth was similar to that in the controls

It is of note that the amount of polyethylene glycol taken by the



Figs 4–7

Fig 4 Eight day tumour transplant in polyethylene glycol treated mouse Note marked lack of proliferation and infiltration (HE × 150)

Fig 5 As Fig 4 Note lack of inflammatory reaction and sharp demarkation between tumour cells (below) and normal tissues (HE × 370)

Fig 6 Eight day tumour transplant in untreated *control mouse* Tumour growth below panniculus carnosus Note Acute cellular inflammatory exudate Irregular band of tumour cells across lower part of figure with necrotic tumour bottom right. (HE × 150)

Fig 7 As Fig 6 Tumour infiltrating muscle layer Note cellular inflammatory exudate and dilated blood vessels in subcutaneous tissues at top of figure (HE × 150)

The surrounding tissues showed little sign of reaction (Fig 5) The blood vessels appeared to be unaffected and no cellular infiltration was present The line of demarkation between tumour tissue and the muscle layer was sharp Few mitoses were seen

The above histological picture was in distinct contrast to that seen in *the control mice at 8 days* which showed marked infiltrative growth and frequent mitoses Where growth was still confined to areas below the panniculus carnosus (Fig 6) its underlying blood vessels which were not prominent in the treated mice were dilated and filled with blood This hyperaemia was accompanied by exudate formation in the space between the muscle layer and the tumour transplant The exudate showed the beginnings of organization with a thread like matrix interspersed with fibroblasts and infiltrated with granulocytes The tumour cells on the surface of the transplant were irregularly arranged and showed frequent mitoses while those deeper in the transplant were necrotic There was no sharp line of demarkation between transplant and normal tissues Instead this area was filled by exudate as described above in which the actively dividing tumour cells mingled

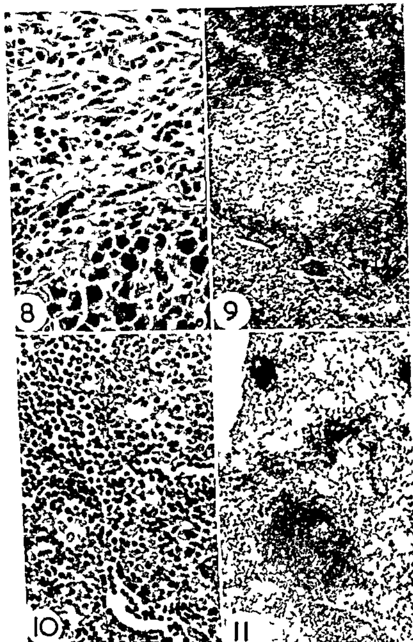
In other areas the inflammatory exudate containing tumour cells was present throughout the muscle layer (Fig 7) In other words the tumour had infiltrated the muscle layer The inflammatory process was also marked in the overlying dermis with dilated blood vessels oedema granulocyte infiltration and scattered tumour cells

In these tumours that had infiltrated the panniculus carnosus the exudate under the muscle layer showed further organization (Fig 8) and now presented a picture of young granulation tissue with fibroblasts and newly formed thin walled blood vessels While these vessels were dilated and blood filled granulocyte infiltration was less marked than in the exudates The tumour cells abutting on to this granulation tissue showed many mitoses In mice treated with *10 per cent polyethylene glycol* and those with *20 per cent glycerol* the histological picture was as in the untreated controls

Lymph Nodes

In the tumour bearing mice treated with *20 per cent polyethylene glycol* the lymph nodes were enlarged at 8 days Follicles were prominent and contained well developed germinal centres with many tingible bodies (Fig 9) The subcapsular sinus was almost completely obliterated being compromised by the diffuse masses of lymphocytes in the outer areas of the follicles Paracortical proliferation was also marked While the cells lining the medullary sinuses were not prominent the sinuses themselves were dilated and tightly packed with mainly small lymphocytes (Fig 10)

The lymph nodes in the untreated tumour bearing controls were also enlarged In contrast to the mice treated with 20 per cent poly



Figs 8–11

Fig 8 As Fig 7 Organized exudate adjacent to tumour cells Note frequent mitoses in neighbouring tumour cells (HE × 370)

Fig 9 Lymph node from mouse with eight day tumour transplant treated with polyethylene glycol Note enlarged follicle with prominent germinal centre and also paracortical proliferation (HE × 150)

Fig 10 As Fig 9 Note masses of small lymphocytes in sinuses (HE × 370)

Fig 11 Spleen from mouse with 8 day tumour transplant treated with polyethylene glycol Note small inactive follicles and lack of cellularity of the pulp (HE × 150)

ethylene glycol these nodes showed only moderate proliferation of the follicles with smaller germinal centres and few tingible bodies Para cortical proliferation was not in evidence The sinuses were dilated lined with rather plump pale staining cells and contained but few lymphocytes

The histological picture in the lymph nodes of tumour bearing mice treated with 10 per cent polyethylene glycol and those treated with 20 per cent glycerol was similar to that in the untreated tumour bearing mice

Spleen

The spleen in mice treated with *20 per cent polyethylene glycol* was atrophic and remarkable histologically for its lack of cellularity The follicles were small and lacked germinal centres The white pulp was represented by a few layers of lymphocytes around the branches of the splenic vessels The red pulp contained few megakaryocytes little extra medullary haematopoiesis and extended right up to the splenic capsule (Fig 11) These findings contrast markedly to that in *untreated non tumour bearing mice* in which follicles are well developed and on occasion contain germinal centres In addition there is usually more white pulp than red and it extends right up to the splenic capsule In *untreated tumour bearing mice* germinal centres are prominent in enlarged follicles The white pulp is extremely cellular and extends to form a band just under the splenic capsule The red pulp is congested and extramedullary haematopoiesis marked at times

In mice treated with 10 per cent polyethylene glycol and those given 20 per cent glycerol the histological findings in the spleen were similar to those described in the untreated tumour bearing mice although the amount of white pulp was reduced in the smaller spleens in both groups

DISCUSSION

The present experiments confirm that oral treatment with polyethylene glycol 4000 will give a significant reduction in subcutaneous tumour growth in both male and female mice As it was previously thought that this reduction in tumour growth might well be due to dehydration per se *i e* that the reduction in tumour size might be merely a reflection of general weight reduction studies were carried out on mice dehydrated with oral glycerol The findings in these mice in which there was a statistically significant positive correlation between tumour size and the degree of dehydration in the treated group as judged from the final body weight supports this hypothesis However although the distribution of both tumour size and spleen weight within the

My thanks are due to *Dr B Hallerarer* of this Institute for suggesting the use of glycerol

glycerol treated group could be shown to be related to the final body weight i e to be influenced by dehydration the extent of tumour growth in the group as a whole was not effected by treatment as the mean tumour size did not differ significantly from that in the untreated controls

In the mice treated with 20 per cent oral polyethylene glycol and also in the untreated mice in all experiments no such correlations were found within the groups That is to say no relationship could be demon strated between tumour growth or spleen weight and the degree of hydration of the host A possible exception was the group of male mice treated with 20 per cent polyethylene glycol in which the positive correlation between tumour growth and body weight was just statistic ally significant However this correlation was obtained from the sum of the two diameters of the tumours No correlation was found to tumour depth As tumour depth is a measurement that is likely to be influenced by the turgidity of the tissues correlation with this factor and possibly spleen weight too as was present in the glycerol treated mice should probably be required before relationship to dehydration can be imputed with any certainty

While dehydration as evidenced by diarrhoea and weight loss was certainly present in the polyethylene glycol treated mice it thus seems that it is unlikely to be the only or even the main factor responsible for the reduction in tumour growth seen in these mice although reduc tion in tumour growth did not occur in its absence

Reduction in tumour growth was only achieved with 20 per cent polyethylene glycol so high dosage appears essential Reduction in tumour growth was also regularly accompanied by reduction in spleen weight The histological picture of splenic atrophy in these mice is in striking contrast to the cellularity usually seen in the spleens of tumour bearing mice whether dehydrated by glycerol or untreated This marked splenic atrophy is reminiscent of that seen under general conditions of stress However the lymph nodes then show a similar atrophic picture In contrast the lymph nodes in these mice were enlarged and the enlargement was due to cellular proliferation both in the follicles which showed hyperactive germinal centres and para cortically Although the lymph nodes in tumour bearing mice not treated with polyethylene glycol were also enlarged lymphocyte pro liferation was by no means so marked particularly paracortically Further in the untreated tumour bearing mice the findings in the spleen and nodes were comparable The dissociation between the find ings in the spleen and lymph nodes in polyethylene glycol treated mice is therefore unusual and needs further investigation before its aetio logy can be discussed with profit

The main finding reported in this paper concerns the difference in the histology of the subcutaneous tumours in polyethylene glycol treated mice and their untreated controls which has been described

in detail From these findings it is clear that subcutaneous growth of this tumour is associated with the presence of an inflammatory reaction in the surrounding normal tissues In the absence of such a reaction infiltrative tumour growth did not occur Expansive growth i e the layering of one cell layer on the other did occur This gave a pattern of rows of cells parallel to the surface of the transplant It seemed that a layer of tumour cells about 10 cells deep could obtain sufficient oxygenation by diffusion for survival but proliferation was not active Deeper than this the transplant showed a uniform necrosis in the absence of bleeding or any other reactive changes This is hardly surprising as there was no sign of vascularization of the transplants at all

The surface of the transplant in these polyethylene glycol treated tumours was sharply demarkated from the normal tissues which appeared to be completely unaffected by its presence there being no oedema inflammatory cell infiltration or changes in the blood vessels The tumour cells on the surface of the transplant are thus surviving but the tumour seems unable to establish any stromal reaction While the cells themselves have previously proven their ability to grow in a malignant fashion and do so again in the controls they do not behave in that way under the present circumstances The initial transient swelling at the injection site in the polyethylene glycol treated females suggests that an inflammatory reaction may have occurred in these mice too before treatment took effect

This failure to establish infiltrative growth could be due to a direct effect of polyethylene glycol on the tumour cells The possibility can not be ruled out at present but it is unlikely as this substance is not toxic to tumour cells in short term suspension (*Hartveit* 1967) and long term intraperitoneal treatment with 1 ml 10 per cent polyethylene glycol 3 times per week does not appear to have adverse general effects on our mice (*Hartveit* 1969)

The present findings suggest rather that it is the host tumour relationship that has been upset by treatment In the control mice growth was infiltrative in type There was no sharp borderline between tumour transplant and the surrounding normal tissues The transition area was filled with a copious acute inflammatory exudate and the blood vessels in the surrounding tissues showed corresponding inflammatory changes

This response is unlikely to be due to a bacterial contaminant in the tumour cell suspension in the control group as the experimental group was injected from the same suspension as the controls on each occasion Capillary permeability increasing factors are known to be present in the ascitic fluid from such tumours (*Thunold* 1965) but in the present case so little tumour ascitic fluid was injected that it is unlikely that the small amounts of these agents present in it could have an effect that was so widespread and so longlasting and in any case the

same amount of fluid was injected in control and treated groups If the fluid is not responsible we are left with the tumour cells themselves (plus any intergral component i e virus they may carry)

In the untreated animals the presence of transplanted tumour cells was followed by the development of an inflammatory response Such cells also elicit an inflammatory response on intraperitoneal transplantation—with the formation of an ascitic tumour in consequence (*Hartveit* 1965 a) The extent of the inflammatory response is related to the number of tumour cells that undergo immunological lysis after transplantation Following tumour cell lysis exudate formation occurs This exudate collects as ascites in which the remaining tumour cells thrive protected from further immunological lysis by the anticomplementary nature of the inflammatory exudate (*Hartveit* 1965 b *Hartveit* 1966)

However if the tumour cells are transplanted subcutaneously the exudate will not have the opportunity to collect to this extent The fluid will tend to diffuse away while the more solid remains will undergo organization This is demonstrated at the edge of the tumours in the present experiment where areas with fresh unorganized exudate were found adjacent to areas where organization was well under way

In contrast to the orderly arrangement of the tumour cells parallel to the surface of the transplant in treated tumours the tumour cells abutting on to this inflammatory exudate surrounding the transplant in the control mice showed no sign of orderly arrangement The tumour cells appeared to be scattered at random throughout the exudate and to be carried with it into the surrounding tissues The nutritional requirements of the tumour cells were obviously amply supplied by this exudate and enough oxygen was apparently present to meet their needs as mitoses were frequent Where organization of the exudate had occurred the tumour cells seemed to have been left high and dry between the newly formed fibroblasts and capillaries Once more their nutritional requirements were obviously satisfied as mitoses were frequent here too

The ability to elicit a stroma and so secure a blood supply thus seems to be dependent on the primary ability to evoke an inflammatory response The present findings suggest that in the absence of an inflammatory response local tumour growth is doomed to be of expansive type In its presence infiltrative growth becomes possible

To achieve an inflammatory response interaction between host and tumour is essential the tumour cells supplying the irritative factor to which the host reacts Failure of either of these processes may be responsible for failure in exudate formation and/or organization

In the polyethylene glycol treated mice the inflammatory response has clearly failed to become established In addition treatment with polyethylene glycol has changed the histological response of both the spleen and lymph nodes to tumour transplantation If as has been

discussed previously the inflammatory stimulus is derived from immunologically injured tumour cells changes in immune response consequent to these histological changes in the reticuloendothelial system need to be taken into consideration

The tumour may fail to elicit an inflammatory response as the host fails to supply the factors *i e* specific antibody and/or complement required for immunological lysis

Polyethylene glycol is known to potentiate immunological lysis *in vitro* It has been suggested that it acts indirectly by forming a loose complex with gamma globulin that in turn traps complement This complement is not fixed in the sense that it is inactivated but may on the contrary be so placed that reaction with antibody is facilitated (see *Hartveit* 1967) If binding of complement occurs intravascularly in polyethylene glycol treated mice it is unlikely that such large complexes could pass into the extravascular space This could lead to extravascular decomplementation As *Willoughby et al* (1969) have recently pointed out complement plays an important part in inflammation be it of specific or non specific type In the present case all the ingredients required for immunological lysis of the tumour cells are normally available in vivo It is tempting to postulate that lack of available complement due to intravascular trapping may be responsible for the lack of inflammatory response round the tumour transplants in the treated mice

The probability arises from these findings that the ultimate means of achieving infiltrative growth may be given to the tumour cells by the host As such it would be an acquired characteristic not an inborn behaviour disorder peculiar to malignant cells

This hypothesis implies that

1 The cell population involved differs antigenically from its host (The potential malignant cell is thus initiated by factors capable of leading to genetic change *e g* chemicals viruses ionizing radiation)

2 The host is capable of responding to the antigenic stimulus with humoral antibody production and of mounting an inflammatory response to subsequent immunological lysis

3 Cell proliferation within the population is capable of outstripping cell death due to immunological lysis

If these conditions are fulfilled the host may be capable of endowing that cell population with the means of infiltrative growth Working with transplanted Ehrlich carcinoma in our untreated mice these conditions are fulfilled The tumour is an allograft to which humoral antibody is formed (*Thunold* 1968) Immunological lysis takes place in the early stages of growth but cell proliferation finally outstrips this lysis (*Hartveit* 1963)

Cellular immunity to tumour growth has received much attention recently (see *Alexander & Fairley* 1967) However experience assures us that immunity to spontaneous tumour growth whether in man or in

animals is a rare exception if it indeed occurs Further *Moller s* (1963) finding that humoral antibody can effectively prevent the recognition of tumour cells by primed host lymphocytes suggests that the cellular response if established may be doomed to failure The latter is supported by the failure of human malignant tumours in general to evoke a local cellular immune response

While cellular immunity has thus failed to establish its place in the physiopathology of tumour growth the probability that humoral immunity may be of aetiological significance has now to be considered

How far conditions similar to those found with the Ehrlich carcinoma prevail in spontaneous tumours remains an open question The finding that increase in tumour size is not directly proportional to the rate of tumour cell proliferation in malignant human neoplasms (see *Refsum* 1968) leads to the assumption that cell death is present here also While it is unusual to find a cellular inflammatory exudate round actively growing tumour tissue in human material the possibility of increased permeability of the surrounding vessels needs further investigation *Burgess & Sylven s* experimental finding (1962) of increased protein content in tumour interstitial fluid coupled with *Cater & Wallington s* (1968) demonstration of the increased sensitivity of newly formed tumour vessels to chemical mediators of inflammation provide additional reasons to investigate this further

SUMMARY

Inhibition of growth of Ehrlich s carcinoma by oral treatment with polyethylene glycol 4000 is accompanied by a marked reduction in the inflammatory response around subcutaneous tumour transplants In the absence of formation of an inflammatory exudate tumour growth was expansive and not infiltrative in type

The lymph nodes in treated animals showed marked proliferation while the spleen was atrophic Such changes were not present in the untreated controls It is suggested that treatment with polyethylene glycol may have upset the immunological balance in host tumour relationship and that subsequent changes in response may be ultimately responsible for the difference in the subcutaneous growth of these tumour transplants

REFERENCES

Alexander P & Fairley G H Cellular resistance to tumours Brit Med Bull 23 86–92 1967

Burgess E A & Sylven B Changes in glucose and lactate content of ascites fluid and blood plasma during growth and decay of the ELD ascites tumour Brit J Cancer 16 98–305 196–

Cater D B & Wallington T B Inflammatory changes in newly formed vessels of carrageenin induced granulomas after systemic 5 hydroxytryptamine bradykinin kallikrein or lysolecithin Brit J Exp Path 49 74–80 1968

Hartveit F Sex differences in the intraperitoneal growth of Ehrlich s ascites carcinoma Acta path microbiol scand *58* 25–32 1963
Hartveit F The immediate cause of death in mice with Ehrlich s ascites carcinoma Acta path microbiol scand *65* 359–365 1965 a
Hartveit F In vitro oncolysis and its inhibition by mouse serum Acta path microbiol scand 65 354–358 1965 b
Hartveit F The inhibitory effect of mouse gamma globulin on the lysis of isologous mouse tumour cells by human serum Acta path microbiol scand *68* 29–36 1966
Hartveit F In vitro potentiation of immune oncolysis by polyethylene glycol and by polyvinylpyrrolidone J Path Bact *94* 200 204 1967
Hartveit F Inhibition of the subcutaneous growth of Ehrlich s ascites carcinoma in female mice by polyethylene glycol 4000 J Path *97* 145–148 1969
Jones F S & Rous P On the cause of the localization of secondary tumours at points of injury J exp Med *20* 404 412 1914
McKelar D L Enhancement of hemolytic activity of complement by polyethylene glycols Proc Soc Exp Biol Med 72 384–385 1949
Moller G Studies on the mechanism of immunological enhancement of tumour homografts III Interaction between humoral isoantibodies and immune lymphoid cells J Nat Cancer Inst *30* 1205–1226 1963
O Meara R A Q & Jackson R D Cytological observations on carcinoma Irish J med Sci No *371* 327–328 1958
O Meara R A Q Fibrin formation and the growth of spontaneous cancers in man Bull de la soc internat de Chir 23 24–29 1964
Refsum S B & Berdal P Cellepopulasjonskinetikk i maligne svulster Tid for Den norske Lægefor no *12b* 1224–1227 1968
Thunold S Permeability increasing effect of cell free ascitic fluid from Bergen A4 ascites carcinoma Acta path microbiol scand *63* 636–637 1965
Thunold S Globulin coating in vivo of Ehrlich s ascites carcinoma cells Transplantation 6 716–727 1968
Vasiliev Ju M The role of connective tissue proliferation in invasive growth of normal and malignant tissues A review Brit J Cancer *12* 524–536 1958
Wheatley D N Ambrose E J & Easty C C Infiltration of intra abdominal organs by ascites tumours Nature (London) *199* 188–189 1963
Willoughby D A Coote E & Turk J L Complement in acute inflammation J Path 97 295–305 1969

Acta path microbiol scand 77 639–645 1969

The Gade Institute Department of Pathology The University Bergen Norway
(Head Prof E. Waaler MD)

THE INHIBITORY EFFECT OF ORAL POLYETHYLENE GLYCOL 4000 ON THE PHAGOCYTIC ACTIVITY OF THE RETICULOENDOTHELIAL SYSTEM, RELATED TO TUMOUR TRANSPLANTATION IN MICE

By

F HARTVEIT

Received 21 vi 69

Polyethylene glycol is a long chain polymer which has the ability of potentiating the action of complement in vitro in both the immune haemolytic (*Mcl tckar* 1949) and immune oncolytic (*Hartveit* 1967a) systems When given orally or intraperitoneally it has been shown to inhibit the subcutaneous growth of Ehrlich s carcinoma in mice (*Hartveit* 1969 1970) Oral treatment with polyethylene glycol 4000 is accompanied by proliferative changes in the lymph nodes with active germinal centres and paracortical proliferation while the spleen remains small and inactive compared to the spleen in tumour bearing mice that have not been given polyethylene glycol

The mode of action of polyethylene glycol on tumour growth in vivo is as yet unexplained though it may be related to its ability to alter the reactivity of complement (see *Hartveit* 1970) At the same time polyethylene glycol s physical character and high molecular weight make it likely that it will be taken up by the reticuloendothelial system The following experiments were set up to see if this were so using estimation of carbon clearance as a measure of the phagocytic ability of the reticuloendothelial system as a whole

MATERIAL AND METHODS

Mice aged 5 months of the closed colony kept at this Institute were used

Polyethylene glycol 4000 was given as a 20 per cent solution in the drinking water ad lib

The tumour used was the Ehrlich ascites carcinoma kept by serial intraperitoneal transplantation in our mice Whole tumour ascites from a 10 day transplant was injected subcutaneously on the back at a dosage of 0 05 ml per mouse

The phagocytic activity of the reticuloendothelial system was determined by

---

Requests for reprints should be addressed to Dr F Hartveit Gades Institutt Haukeland Sykehus, 5000 Bergen Norway

carbon clearance assessed by direct microscopical evaluation of blood samples (*Hartveit et al* 1967b) A carbon dosage equivalent to 8 mg/100 g body weight was used

Experimental Procedure

The carbon clearance time was determined in male and female mice

1) in the absence of treatment
2) following treatment with oral polyethylene glycol
3) following tumour transplantation alone.
4) following tumour transplantation and treatment with oral polyethylene glycol starting from the time of transplantation

At stated intervals see Figs 1 and 3 the clearance time was recorded and the mice were killed The spleens were removed fixed in formalin and later weighed after standing for one hour in the air

RESULTS

Treatment with Polyethylene Glycol per os

The clearance time increased in both sexes when the mice were given polyethylene glycol to drink (Fig 1) The positive correlation between clearance time and days of treatment was statistically significant in both sexes $0.001 > P$ $r ♂ = 0.7977$ $r ♀ = 0.7538$

The spleen weight on the contrary decreased in both sexes on treatment with polyethylene glycol (Fig 2) The negative correlation between spleen weight and days of treatment was statistically significant in both sexes $0.001 > P$ $r ♂ = -0.7246$ $r ♀ = -0.7625$

There is thus in both sexes a negative correlation between clearance

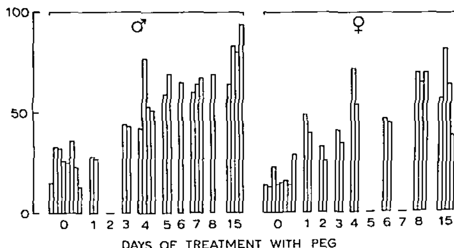


Fig 1

The individual clearance times in untreated mice (0 days) and mice treated with polyethylene glycol (PEG) related to days of treatment

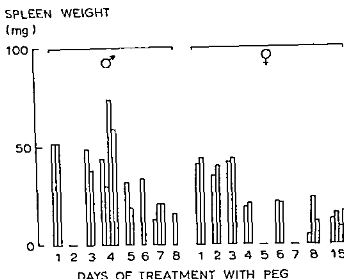


Fig 2

The individual spleen weights in polyethylene glycol (PEG) treated mice related to days of treatment

time and spleen weight r ♂ = — 0 6472 (*0 01 > P > 0 001*) r ♀ = — 0 7625 (*0 001 > P*)

Tumour Transplantation

The *clearance time* following subcutaneous tumour transplantation was similar to that in untreated mice (see day 0 Fig 1) at 3 days but then dropped (Fig 3) The overall difference i e reduction in clearance time from that in the untreated mice was statistically significant ♂ *0 01 > P > 0 001* ♀ *0 02 > P > 0 01*

The spleen weight showed a marked rise by the 8th day followed by a drop both rise and fall being more pronounced in the males than in the females (Fig 4)

Treatment with Polyethylene Glycol and Tumour Tranplantation

The clearance time fell slightly in both sexes with time (Fig 3) but all in all did not differ significantly from that in untreated mice (see day 0 Fig 1)

The spleen weight showed the same pattern i e a rise by day 8 followed by a fall in both sexes as was seen in the mice given tumour alone (Fig 4) However although the pattern was similar the spleens were much smaller The overall means ± S D in the mice given tumour alone were ♂ 131 ± 128 mg ♀ 112 ± 59 mg while the corresponding figures for those given tumour plus polyethylene glycol were ♂ 43 ± 18 mg and ♀ 53 ± 22 mg

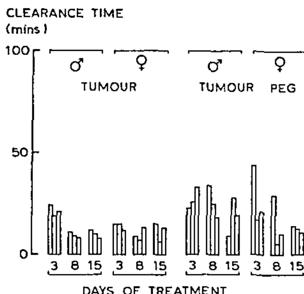


Fig 3

The individual clearance times in mi e treated with subcutaneous tumour and in mice treated with tumour and polyethylene glycol (PEG) related to days of treatment

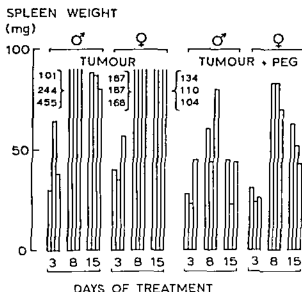


Fig 4

The individual spleen weights in mice treated with sub utaneous tumour and in mice treated with tumour and polyethylene glycol (PEG) related to days of treatment

DISCUSSION

The present experiments demonstrate that mice given polyethylene glycol 4000 per os become progressively less able to remove carbon particles from their blood stream. That is to say the phagocytic activity of the reticuloendothelial system is depressed.

This reduction in phagocytic activity is accompanied by a reduction in the size of the spleen. As the spleen contains a high proportion of the cells responsible for the phagocytosis of carbon particles in the body this reduction in splenic weight is probably to a great extent responsible for the delayed clearance time recorded.

A similar reduction in spleen weight and in carbon clearance has also been found in mice given polyethylene glycol 4000 intraperitoneally (personal observation). It is thus likely that the substance is absorbed when given per os and the possibility of an indirect effect on the reticuloendothelial system can probably be excluded. Blockade of the phagocytes by polyethylene glycol can well be expected due to the physical characteristics of the molecule. However although the experiment demonstrates that phagocytic activity is decreased it cannot tell us whether the reduction is in fact due to prior blockade of the phagocytes to a reduction in their number or to a mixture of these two processes.

In untreated tumour bearing mice the phagocytic activity of the reticuloendothelial system was increased. This increase was accompanied by a gross increase in spleen weight as has been reported previously (*Hartveit* 1966). The increase was maximal at 8 days after transplantation and then dropped. This finding is also in keeping with previous studies from this Institute (*Thunold* 1968). For the reasons mentioned above it is reasonable in this case to attribute the reduction in carbon clearance to increased phagocytic capacity of the spleen.

The tumour bearing mice treated with polyethylene glycol showed clearance times and spleen weights within the range of normal (Figs 3 and 4) i e between these two extremes. This suggests that two opposing forces have been at work and that the stimulating effect of transplantation cancelled out the depressive action of polyethylene glycol.

While these clearance times are not surprising viewed in the light of the spleen weights recorded they call for comment when the previous finding that tumour growth is reduced in polyethylene glycol treated mice is also taken into consideration.

The reactivity of the lymphoid and phagocytic elements of the reticuloendothelial system are usually assumed to go hand in hand. However in the present experimental situation there is reason to believe that this may not be so as concommitant lymph node hyperplasia and splenic atrophy have been reported in tumour bearing mice treated with oral polyethylene glycol (vide supra).

This odd combination of findings was accompanied by a reduction

in tumour growth compared to that in untreated tumour bearing mice in which proliferation of the lymphoid elements was recorded in both the lymph nodes and spleen The present work shows that in the case of untreated tumour bearing mice such increased activity was accom panied by increased activity of the phagocytic elements Stimulation of the lymphoid elements in the lymph nodes and spleen in the presence of stimulated phagocytosis thus appears to be accompanied by better tumour growth than stimulation of the lymphoid elements in the lymph nodes and depression of the phagocytic elements of the reticulo endothelial system However this is obviously not the whole story as qualitative differences in the histology of the lymphoid tissue reaction were also recorded and need further study

The role of the reticuloendothelial system in relation to tumour growth is variously described in the literature For example *blockade* by trypan blue will permit the growth of tumours in genetically in compatible hosts (*Ludford* 1931 *Andervont* 1936) *Depression* by cortisone may have a similar effect and has been extensively used in studies on xenotransplantation (see *Toolan* 1953) *Depression* or '*exhaustion* in the course of tumour growth has been evoked to ex plain the survival of antigenic tumours in their host of origin (see *Alexander & Fairley* 1967) and *inactivation* by irradiation is also a common experimental tool *Stimulation* by BCG vaccine on the other hand is said to have an inhibitory effect on tumour growth (*Old et al* 1959)

While the findings quoted may be clear cut they are difficult to interpret as the reticuloendothelial system as a whole is considered and not the interplay of its various elements With the recent recogni tion of the significance of paracortical versus follicular proliferation of the lymphoid elements (*Turk & Oort* 1967) the role of thymic dependence (*Parrott et al* 1966) and the part played by macrophages in the initiation of immune responses (*Dumonde* 1967) much of the pre vious work on this system is in urgent need of revision particularly as far as its relationship to tumour growth is concerned

SUMMARY

Oral treatment with polyethylene glycol 4000 was found to decrease the phagocytic activity of the reticuloendothelial system in mice as measured by carbon clearance This is probably related to the decrease in spleen weight also seen in these mice In tumour bearing mice (sub cutaneous Ehrlich carcinoma) given polyethylene glycol the phagocytic activity and spleen weight was similar to that in normal mice However as untreated tumour bearing mice showed a decrease in carbon clear ance and an increase in spleen weight these apparently normal values are probably the result of a combination of stimulation and depres sion of the reticuloendothelial system

REFERENCES

Alexander P & Fairley G H Cellular resistance to tumours Brit Med Bull 23 86-92 1967

Anderson H B The influence of trypan blue upon the resistance of mice to transplantable and induced tumours Publ Hlth Rep Wash 51 591-600 1936

Dumonde D C The role of the macrophage in delayed hypersensitivity Brit Med Bull 23 9 14 1967

Hartveit F The growth of Ehrlich s ascites carcinoma in C3H mice and in mice of an unrelated closed colony Splenomegally following intraperitoneal transplantation Brit J Cancer 20 825-830 1966

Hartveit F In vitro potentiation of immune oncolysis by polyethylene glycol and polyvinylpyrrolidone J Path & Bact 94 200-204 1967a

Hartveit F Andersen K Flatabo G & Gundersen S Measurement of carbon uptake by the reticulo endothelial system Acta path microbiol scand 71 ?2-5?8 1967b

Hartveit F Inhibition of the subcutaneous growth of Ehrlich s ascites carcinoma in female mice by polyethylene glycol 4000 J Path 97 145-148 1969

Hartveit F Oral polyethylene glycol s inhibitory effect on the subcutaneous growth of Ehrlich s carcinoma in mice and on the local inflammatory response to the tumour Acta path microbiol scand 77 6?3-638 1969

Ludford R J Resistance to the growth of transplantable tumours I The influence of vital staining on induced resistance Brit J Exp Path 12 108-113 1931

McKelvar D L Enhancement of hemolytic activity of complement by polyethylene glycols Proc Soc Exp Biol Med 7? 384-385 1949

Old L J Clarke D A & Benacerraf B Effect of Bacillus Calmette Guérin infection on transplanted tumours in the mouse Nature London 184 291-29? 1959

Parrott D M V de Sousa M A B & East J Thymus dependent areas in the lymphoid organs of neonatally thymectomised mice J exp Med 123 191-203 1966

Thunold S Local cellular reactions and splenic weight changes in mice with Ehrlich s ascites carcinoma Acta path microbiol scand 7 54?-55? 1968

Toolan H W Conditioning of the host J nat Cancer Inst 14 745-764 1953

Turk J L & Oort J in Germinal centres in immune responses pp 311-316 ed Cottier et al Heidelberg New York 1967

Acta path microbiol scand 77 646–652 1969

Department of Pathology The Finsen Institute Copenhagen Denmark
(Head Johs Clemmesen D M Sc)

A SIMPLE MICRO CINEMATOGRAPHIC SYSTEM

By

RYAN LINNEMANN and VAGN BREMERSKOV

Received 28 vi 69

During micro cinematographic observation of mammalian cells it is usually significant to keep temperature constant since variation will influence synthetic activity in the cells examined A variety of micro cinematographic systems have been constructed for this purpose (1) of which most are rather complicated and expensive The systems on record (1) appear all to have been based on incubators surrounding the microscope more or less completely Most such arrangements are space consuming and difficult to operate making the microscope useless for other purposes Temperature recordings tend to be incorrect if made in the incubator and not in the cell chamber itself and consequently it is not feasible to measure changes in the chamber temperature *e g* during exposure to transmitted light As this communication reports this may cause serious disadvantages

ELEMENTS

Microscope Carl Zeiss photomicroscope I (light source 6 V 60 W Wolfram) equipped with phase contrast condensor Z VII and standard phasecontrast objectives

Culture chamber The chamber resembles in various ways the Prior tissue culture chamber as obtained from Fa Bie & Berntsen Copenhagen

It consists of two standard microscope cover glasses separated by a O section silicone rubber ring These were originally contained in an aluminum housing but in our modification replaced by a stainless steel housing which by pressure on the silicone ring renders the chamber air tight and is resistent to the cleaning medium RBS 25 Introduction of cell cultures and/or medium is made through injection needles piercing the housing and the silicone rubber ring In the same way it is possible to maintain a constant gas flow through the chamber The chamber is placed on top of a Carl Zeiss heating stage for temperatures ranging from 35 –43 C (86 –109 4 F) Originally the stage was equipped with a reading thermometer and temperature was regulated manually Since temperature variation was found to exceed the permissible the culture chamber was equipped with a built in contact thermometer (Jacob Glasteknik Copenhagen) for automatic temperature regulation Controlled in this way the temperature of the steel housing was set on 38 5 C

Electronic timer For the exposure of the culture chamber to transmitted light with preselected intervals an electronic timer was constructed The diagram of the timer is shown in Fig 3

An astable multivibrator gives of voltage peaks in series which slowly discharges

---

Supported by grants from *The Danish National Anti Cancer League* and from *The Daell Fonden*

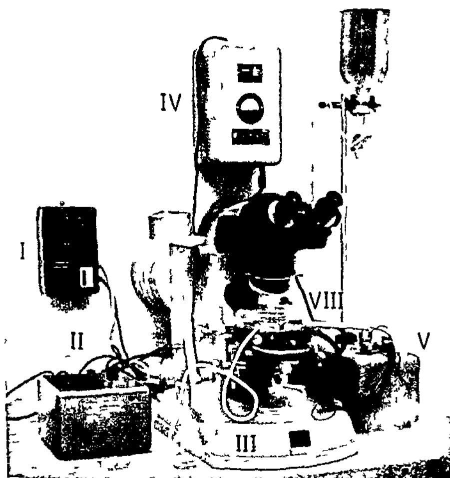


Fig 1 a
A photographic presentation of the microscope m unte l as micro-cinematograph

the condensor on a large RC circuit (50 Mω 64 μF Tantal) The cathode of a diode i connected to the RC circuit and the anode to a variable voltage divider As soon as the cathode potential exceeds the negative preselected level at the anode the diode becomes conducting trigging the m nostable multivibrator via a con densor So the multiv l ator is kipped relea ing a relav connected as collector load in one of the tran istors A variable RC rcuit connected to the base of this transistor keeps the m nostable multivibrator in this po ition for a preselected interval An off conta t at th relay triggers the Carl Zeiss photo timer Another contact charges the RC ircuit which once m re is slowly lischarged By this arrangement frames can le tak n once every 1 10 20 60 nd 120 minutes

METHODS

A schematic presentati n f the test arrangement used for temperature recordings fr m the interior of th ell hamber is sh wn in Fig 4

An iron-constantan th feeler was intr duced into the cl amber through a

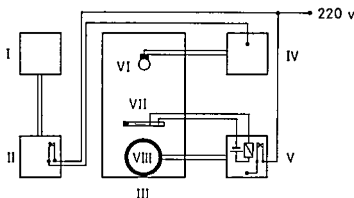


Fig 1 b

The electric wiring of the system I Electronic timer II Carl Zeiss phototimer III Carl Zeiss photomicroscope I IV Power supply for the wolframlamp (6 V 60 W) V Power supply for the heating stage VI Wolframlamp VII Contact thermometer VIII Heating stage The relay contact on the exposure relay cuts out the power supply for the wolframlamp The relay circuit controlling the power supply of the heating stage is cut out by the contact thermometer The electric timer switch on the exposure contact of the phototimer

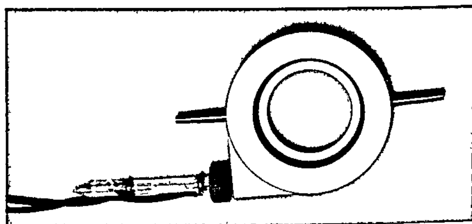


Fig 2

Photographic presentation of the culture chamber The chamber is made of stainless steel The top can be screwed off A 32 mm diameter circular glass cover slip is placed in the base of the chamber A silicone O ring is then placed on it and needles (1 2 × 40 mm) to carry the gas flow and/or act as an air leak when the chamber is filled or emptied is inserted through the two small holes in the base of the housing and through the O ring which is supported meanwhile with the tip of a pair of forceps Another cover slip is then placed on top of the O ring followed by the chamber top which is lightly screwed down to contact with the cover slip

13 gauge needle In order to keep the assay as close to normal conditions as possible the chamber was filled with Eagle MEM medium As reference temperature an ice bath equilibrated for at least 2 hours in a thermo bottle was used The potential provided by the thermo feeler was amplified 1000 times by Gleichstroms Messverstarker KNICK model MV and led to a Honeywell model J12M recorder Two serially connected quicksilver cells (1 35 V) were stored in an icebath and inserted between

Fig 3

Diagram of the electronic timer For details see elements

Fig 4

The test arrangement for temperature recordings from the interior of the culture chamber I Recorder II Variable potentiometer III Quick silver cells IV Amplifier V Ice bath VI Iron constantan thermofeeler VII Heating stage

the amplifier and the recorder through a 100 kohm variable potentiometric voltage divider This was done in order to depress the signal All components were screened and connected to electrical earth except the recorder mass which was connected to ground

RESULTS

First the average temperatures along the diameter of the culture chamber were recorded For this purpose the diameter was divided in 1 mm sections Measurements were performed corresponding to each of these sections In consequence of the heating of the chamber through the surrounding circular housing the temperature measured at any point will represent that of all points at the same distance from the perifery Secondly the temperature variations in each individual point were recorded The results are shown in Fig 5

It is seen that the temperature gradually increases from the centre toward the perifery of the chamber The total variation is equal to 1 08 C However it should be mentioned that the central 6 mm are kept constant on 37 C The figure further shows that the temperature variation at the perifery is $\pm$ 0 05 C (corresponding to the contact

will effectively reduce the light induced temperature variations so that the full value of a strict temperature controlled micro cinematographic system is attained The proposed system furthermore has the advantages of beeing space saving easily operated and inexpensive In comparison with the price of about $ 11 000 for a complete micro cinematograph the cost of the present system besides ca $ 4 500 for a photomicroscope will not exceed $ 500 Another advantage is that its flexibility (dismounting is done by loosening a single screw) permits the use of the microscope for other purposes

SUMMARY

The construction of a simple spacesaving and inexpensive cinematographic system is reported Transmitted light causes significant temperature variations in the culture chamber The importance of such variations is briefly emphasized The temperature variations can be decreased by the insertion of a heat absorber between the light source and the condensor

REFERENCES

1 Cinemicrography in cell biology Ed George G Rose Acad press New York 1963

Acta path microbiol scand 77 653–675 1969

Ullevål Hospital Department of Pathology University of Oslo Oslo Norway
Head Kristen Arnesen M D

SPONTANEOUS AND EXPERIMENTAL THROMBOSIS IN THE MOUSE PLACENTA

By

NARVE MOE

Received 13 vi 69

In a previous study it was shown that the deposits on the syncytium of the normal human placenta are maternal fibrin thrombi formed from platelet thrombi (17) Whether the platelet aggregates are formed on the wall of the maternal blood space or whether they are primarily formed in the flowing blood and thereafter attach to the vascular lining could not be decided The particular pattern of blood flow in the intervillous space was assumed to be responsible for placental thrombosis the high values of coagulation factors generally and locally and the reduction of fibrinolytic activity during pregnancy were pointed to as contributing factors (17) The relative importance of these three sets of thrombogenic factors could not be clearly defined

In the present study the placentas in 16 days pregnant mice were examined Though the placenta in mouse differs from that in human subjects there are certain similarities with regard to structure and circulatory flow pattern One should therefore expect to find thrombi also in the normal mouse placenta In an attempt to enhance the formation of thrombi animals were made hypercoagulable by ellagic acid which has been found to activate factor XII (Hageman factor) (20)

The purpose of the present study is to explore the following problems

1 Is thrombosis within the maternal blood channels a normal feature even of the mouse placenta? If so where are the thrombi located? How are they formed and what is their fate?

2 If there is a tendency to thrombosis in the mouse placenta is it caused by a generalized thrombogenic propensity during pregnancy or does it depend on local factors?

Requests for reprints should be addressed to *Narve Moe* Ullevål Hospital Department of Obstetrics and Gynecology Josefinesgt 30 Oslo 3

The determination of fibrinogen by dr *H C Godal* Ullevål Hospital Haematological Research Laboratory Dept IX University of Oslo is gratefully acknowledged

MATERIALS

Animals Altogether 205 virgin female albino mice were used They were 55–77 days old and weighed 25–33 g (mean 28.1 g) The animals were kept under artificial light in plastic cages the floor of which was covered by saw dust They were fed a standard diet (SIFF Norwegian standard stock diet No 1 mice and rats) and tap water *ad libitum* Animals taken out for mating were kept single and the males were put in with the females in the morning After 24 hours the males were removed That day was designated day one of pregnancy if the animal one week later showed increasing weight All experiments were performed at day 16 of pregnancy The body weight was then 39–55 g (mean 46.0 g)

Ellagic acid Supplied by K and K Lab Inc New York U S A The ellagic acid was dissolved in buffered saline to a concentration of 4×10^{-4} M as described by *Nordoy & Chandler* (18)

Buffered saline A 0.9 per cent solution of NaCl in distilled water was buffered with 10 per cent tris (hydroxymethyl) aminomethane HCl 0.15 M to a final pH 7.2 and used as diluent

Heparin Heparin 5 per cent AL Oslo Norway containing 5000 I U per ml was used In all experiments it was diluted in buffered saline to a final concentration of 50 I U per ml

METHODS

Anaesthesia The animals were anaesthetized by intraperitoneal injection of *Nembutal Veterinary* (Abbott Lab Ltd Queenborough Kent England) 5 mg per 100 g body weight

Experimental procedure The number of pregnant animals and the duration of the experimental period in the various groups of animals are summarized in Table 1

TABLE 1

Experimental Groups

Injection given	No of animals	No of placentas	Time of sacrifice after last injection
Pregnant			
Untreated	10	82	–
Ellagic acid	10	78	3 min
Ellagic acid	10	100	10 min
Ellagic acid	10	97	24 hrs
Heparin and ellagic acid	5	45	10 min
Heparin and ellagic acid	5	38	24 hrs
Buffered saline	5	45	3 min
Buffered saline	5	40	10 min
Buffered saline	5	50	24 hrs
Non pregnant			
Untreated	5		–
Ellagic acid	5		3 min
Ellagic acid	5		10 min
Ellagic acid	10		24 hrs
Buffered saline	5		3 min
Buffered saline	5		10 min
Buffered saline	5		24 hrs
	105	575	

Grained barley 35 per cent grained oat 15 per cent grained wheat 12 per cent soya meal 12 per cent herring meal 10 per cent grass meal 5 per cent dried skimmed milk 10 per cent salt and mineral and vitamin mixtures 1 per cent

Ellagic acid was given into the tail vein in a dosage of 1 ml (4×10^{-4} M) per 100 g over a period of 30 seconds

In some experiments heparin in a dosage of 10 I U per 100 g body weight was given into the tail vein in a course of 10 seconds Immediately after the administration of heparin ellagic acid was injected through the same needle over a period of 30 seconds

In control animals a comparable volume of buffered saline was substituted for the solution of ellagic acid

No unintended deaths occurred among the animals

The organs for microscopical examination were removed in the anaesthetized animal for immediate fixation In the group examined 24 hours after ellagic acid the animals were re anaesthetized and the organs removed for fixation

Fixation and staining methods for light microscopy All placentas kidneys liver lungs and heart from the animals were fixed over night in formaldehyde mercuric chloride acetic acid (8 per cent formaldehyde with 5 per cent mercuric chloride To this solution was added 5 per cent glacial acetic acid before use) The specimens were embedded in paraffin and cut at 5 μ The placentas were cut transversely through the middle part One section from each organ was stained routinely with Masson s haematoxylin erythrosin saffron method (22) Selected sections were stained with Lendrum s Martius scarlet blue method (MSB) (15)

Examination by light microscopy All sections were examined without knowledge of the treatment given to the animal in question

Fixation and staining method for electron microscopy Two animals sacrificed 10 minutes after treatment with ellagic acid and buffered saline respectively were used Tissue blocks from one placenta in each animal were fixed for 1½–2 hours in chilled isotonic 1¼ per cent glutaraldehyde in M/20 phosphate buffer (pH 7 4) and post fixed for 1¼ hour in 1 per cent isotonic osmic tetroxide (3) and embedded in Epon 812 One micron thick sections were cut on a Huxley ultramicrotome (Cambridge Inst Co) and stained with toluidine blue for orientation by light microscopy Suitable areas were selected and ultrathin sections stained with uranyl acetate and lead citrate (21) They were examined in a Zeiss EM 9 electron microscope

Bleeding time 3 minutes after the injection of ellagic acid or buffered saline the tail of the anaesthetized mouse was cut with a sharp razor blade approximately mm from the tip after it had been prewarmed in 0 9 per cent NaCl at 37 C for one minute. The animal was placed on a horizontal surface with the tail hanging down into a bath containing 0 9 per cent NaCl at 37 C and the time was measured from the moment the tail was cut until the bleeding into the saline had stopped for one minute (18)

Whole blood clotting time Immediately after the bleeding time had been measured the tail was cut near the root and a capillary pipette was filled with blood At short intervals a small piece of the pipette was broken off The clotting time was recorded as the time from the filling of the pipette until the first appearance of a fibrin thread

Fibrinogen Anaesthetized animals were decapitated and 0 9 ml of blood were allowed to flow into a plastic tube containing 0 1 ml of citrate anticoagulant Fibrinogen was measured by the method of *Schneider* (24) modified by *Hjort* (7)

Statistical methods Statistical evaluation of group differences was made by the Wilcoxon rank test (26)

THE LABYRINTHINE PLACENTA IN THE MOUSE (1)

The labyrinth is the major location of exchange of metabolites between mother and foetus In this part of the placenta the foetal and maternal vessels intermingle The foetal vessels are of capillary size and lined by endothelium They are surrounded by small ramifying maternal blood channels the trophoblastic tubules (Fig 1)

The layer of the placenta which is closest to the decidua basalis is called the giant cell layer and corresponds to the floor of the human placenta It is a plate of cells arranged in irregular sheets and cords The cells are of two types A large type the giant [illegible]ls the cytoplasm of which is dense and runs out in thin long processes There i[illegible]ly one large nucleus The other type of cells is smaller though the size and [illegible]aries somewhat Their cytoplasm is basophilic and con-

tains abundant glycogen The nuclei are small and dark These cells are usually arranged in clusters

The decidua of the mouse uterus is relatively scant compared with that of the human uterus

The uterine and decidual arteries unite into a single central artery for each placenta The artery passes through the placenta and ends in the placental roof" in a system of lacunae lined with syncytium (lacunae of the roof") From these the blood passes through the narrow trophoblastic tubules which are lined by a layer of syncytiotrophoblast (the tubular area) The trophoblastic tubules empty into wide lacunar spaces of the giant cell layer mostly lined with endothelium like cells (lacunae of the giant cell layer) The maternal blood is drained through endothelium lined maternal veins which leave the placenta peripherally

RESULTS

The following intravascular bodies of thrombotic nature were distinguished

1 *Loosely Packed Platelet Aggregates*

Loosely aggregated clumps of granular platelets which appeared to be either floating free in blood or occasionally loosely attached to the wall of the maternal blood channels Such aggregates frequently occurred in the uterine veins (Fig 2) in the lacunae of the roof and in the lacunae of the giant cell layer

Loosely arranged platelet aggregates were also regularly observed in the small pulmonary vessels and occasionally in the right ventricle and the coronary arteries of the heart and in the liver veins a few were seen in the kidney vessels

Since the significance of these aggregates is uncertain they have not been included in the following analysis

2 *Dense Platelet Aggregates*

Densely packed platelet aggregates formed more well defined bodies composed of platelets in intimate contact with each other (Figs 3 and

---

Figs 1–4

Fig 1 Transverse section through the middle part of a labyrinthine placenta in a 16 days pregnant mouse LR lacunae of the roof T tubular area G giant cell layer LG lacunae of the giant cell layer which often extend into the tubular area D decidua M myometrium U umbilical vessels Arrow placental artery HPS × 30

Fig 2 Loosely packed platelet aggregates in a decidual vein Some of the aggregates are in contact with the endothelium MSB × 500

Fig 3 A dense platelet aggregate is occluding a trophoblastic tubulus in the tubular area Syncytial nuclei are pycnotic MSB × 500

Fig 4 Two neighbouring lacunae of the giant cell layer An apparently freely floating platelet aggregate is seen in the smaller lacuna in the lower part of the picture Many of the platelets are swollen and the darker outline indicates traces of fibrin (arrow) The larger lacuna in the middle of the picture contains a thrombus mostly composed of fibrin and apparently attached to the vascular lining Red stain of fibrin appears dark grey MSB × 500


LR
U
T
LG
G
D
M
1
2
3
4

4) Single platelets were often difficult to distinguish In some aggregates the platelets were granular In others many of the platelets appeared to be swollen and had an empty interior (Fig 4) Traces of fibrin at the periphery could be distinguished in some of the aggregates

3 *Platelet Fibrin Masses*

Platelet aggregates with distinct fibrin membranes at or near the periphery of the individual platelet aggregates (Figs 12 and 13)

The dense platelet aggregates and the platelet fibrin masses either appeared to be floating free in the blood stream or they seemed to be attached to the vessel wall

4 *Fibrin Thrombi* (Figs 4 7 8 9 10 and 11)

Thrombi predominantly composed of densely packed fibrin fibrils often with what appeared to be remnants of platelet masses in between the fibrin strands Occasionally eosinophilic masses were observed in which no fibrillar structure could be distinguished except near the surface The interior of these masses was either hyaline or granular

The fibrin thrombi were always attached to the vessel wall as mural thrombi

5 *Hyaline Microthrombi* (Fig 6)

In the small trophoblastic tubules structureless bodies with an appearance similar to that of hyaline microthrombi (25) were seen in a few cases

Thrombosis in the Placenta and in the Myometrial Vessels of Untreated Mice

Dense platelet aggregates platelet fibrin masses and fibrin thrombi were observed in all parts of the maternal vessels of the placenta except the placental artery they were never observed in foetal vessels In the myometrium they were seen in the veins and not in the arteries

Dense platelet aggregates were seen in small trophoblastic tubules of all placentas They were preferentially located to the upper part of the tubular area Most of the dense platelet aggregates occluded the lumen (Fig 3) The cytoplasm of the syncytium bordering the occluded tubules appeared occasionally to be shrunken and the nuclei showed pyknosis or karyorrhexis (Fig 3)

Dense platelet aggregates were occasionally seen in the lacunae of the roof and in the lacunae of the giant cell layer (Fig 4) More rarely they occurred in the uterine veins (Fig 5) In most instances these dense platelet aggregates were floating free although sometimes they appeared to be in contact with the vascular lining without occluding

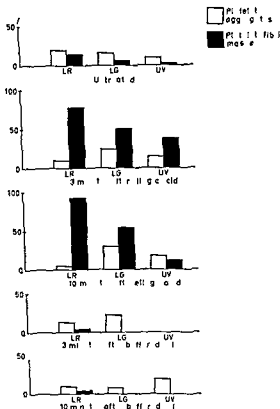


Fig 5

[T]he frequency of apparently freely floating dense platelet aggregates and platelet [f]ibrin masses found in the lacunae of the roof (LR) lacunae of the giant cell layer (LG) and uterine veins (UV) (mean percentage of placentas)

the lumen The lining cells at the sites of contact most frequently seemed unaltered

Platelet fibrin masses were seen in the lacunae of the roof in some cases More rarely they were found in the lacunae of the giant cell layer or in the myometrial veins (Fig 5)

Fibrin thrombi were regularly observed in the lacunae of the roof (Figs 7 and 8) Sometimes they extended a short distance into the proximal end of the tubuli but not in the remaining part of the tubular area Fibrin thrombi were frequently seen in the lacunae of the giant cell layer (Fig 9) and in the uterine veins (Figs 10 and 11) as well

In the lacunae of the roof the fibrin thrombi were regularly located to curved parts or small pockets (Fig 7) The underlying syncytium was occasionally altered as described above (Fig 8)

Underneath fibrin thrombi in the lacunae of the giant cell layer the endothelium like cells were sometimes lacking and fibrin thrombi were in direct contact with trophoblastic cells Occasionally the fibrin

appeared to merge with similar masses between the cells (Fig 9) In the myometrial veins the endothelium underneath the thrombi was often missing (Figs 10 and 11) and the fibrin of the thrombi was continuous with fibrin like masses between the smooth muscle cells (Fig 11)

Alterated lining cells not associated with thrombotic materials were rarely seen

In the placenta there was no invasion of leucocytes into the thrombi and signs of organization were not observed Clear morphological evidence of fibrinolysis was lacking Occasionally the surface of the thrombi was partly or totally covered by syncytium (Fig 8)

In contrast to the situation in the placenta leucocytes were often observed within the thrombi in the uterine veins (Fig 10) Regularly endothelium covered parts of these thrombi (Fig 11)

Thrombosis in the Placenta and the Myometrial Vessels after Injection of Ellagic Acid

Sacrifice at 3 and 10 Minutes after Injection of Ellagic Acid

Fig 5 shows the frequency at which platelet fibrin masses were seen in the different areas of the maternal blood channels (dark columns) The percentage of placentas within each mother animal showing platelet fibrin masses in each location was determined From these values mean percentages were calculated for each location within each experimental group

In animals killed 3 and 10 minutes after injection of ellagic acid there was a marked increase in the number of platelet fibrin masses compared with untreated animals and those treated with buffered

---

Figs 6–11

Fig 6 Hyaline microthrombus (arrow) in trophoblastic tubulus in upper part of labyrinth stains as fibrin (red dark grey in picture) MSB × 500

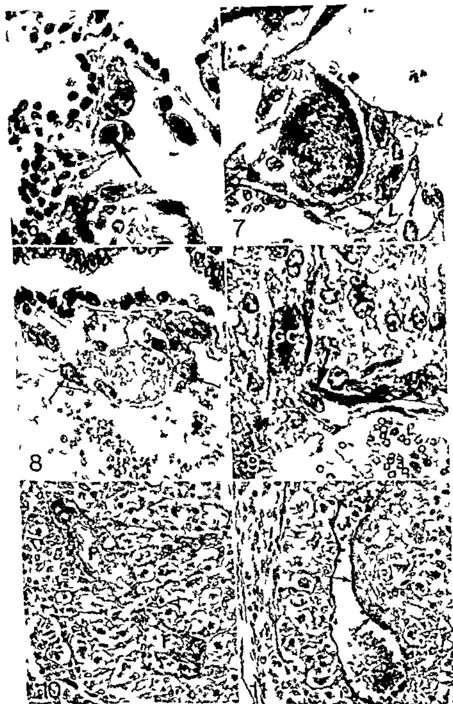
Fig 7 Fibrin thrombus in a pocket of the lacunae of the roof Labyrinth (L) to lower right HPS × 500

Fig 8 *Fibrin thrombus in a lacuna of the roof The syncytium (arrows) is in* part covering the thrombus The nuclei of the syncytium at the base of the thrombus are partly fragmenting HPS × 500

Fig 9 Thrombus mostly composed of fibrin in a lacuna of the giant cell layer The endothelium like cells are missing and masses with the appearance of fibrin (arrows) are seen between the trophoblastic cells GC giant cell HPS × 500

Fig 10 Uterine veins with fibrin thrombi (F) Note several leucocytes within thrombi The endothelium underneath the thrombus is partly lost HPS × 200

Fig 11 Fibrin thrombus in uterine vein The endothelium underneath the thrombus is lost Instead endothelial cells are partly covering the thrombus (arrows) HPS × 200


7
GC
8
10
11

saline The differences between the ellagic acid groups and the untreated group and between the ellagic acid groups and the buffer groups are significant for each location The platelet fibrin masses were located exclusively to the lacunae (Figs 12 13 and 14) and the uterine veins Both in untreated and ellagic acid treated animals platelet fibrin masses were most frequently seen in the lacunae of the roof less frequently in the lacunae of the giant cell layer and still less frequently in the uterine veins However the significance of this apparent down stream decrease in frequency is difficult to estimate since in most cases the lacunae of the roof covered a greater section area than the lacunae of the giant cell layer and the uterine veins

In both lacunar areas platelet fibrin masses were somewhat more frequent in the animals killed 10 minutes after ellagic acid injection than in those killed after 3 minutes whereas the reverse relation was seen in the uterine veins None of these differences are statistic ally significant

Most platelet fibrin masses appeared to have been floating free and were surrounded by erythrocytes (Fig 12) a few seemed to be in contact with the syncytium endothelium or mural fibrin thrombi The size of the platelet fibrin masses and the amount of fibrin asso ciated with the platelets had increased from 3 to 10 minutes after ellagic acid injection in all areas (Fig 13) Ten minutes after the ellagic acid injection the lumen of the lacunae of the roof and of the giant cell layer were nearly occluded by platelet fibrin masses (Fig 14) in some cases

---

Figs 12–17

Fig 12 Three minutes after injection of ellagic acid A mass composed of platelets and fibrin appears to be floating freely in a lacuna of the roof HPS × 500

Fig 13 Ten minutes after injection of ellagic acid Large platelet fibrin mass in lacuna of the roof HPS × 500

Fig 14 Ten minutes after injection of ellagic acid A lacuna of giant cell layer is nearly occluded by a platelet fibrin mass Arrows red blood cells HPS × 200

Fig 15 24 hours after injection of ellagic acid The labyrinth is necrotic and the lacunae and tubuli are dilated and packed with red blood cells Interstitial bleeding has taken place HPS × 200

Fig 16 24 hours after injection of ellagic acid Necrosis of the placenta The la cunae of the giant cell layer are dilated and filled with platelet fibrin mas ses There is leakage of blood cells and fibrin into the junctional zone (arrow) HPS × 75

Fig 17 24 hours after injection of ellagic acid Thrombus mostly composed of platelets and fibrin in uterine vein Note the absence of endothelial cells underneath the thrombus and the presence of leucocytes within the throm bus HPS × 200

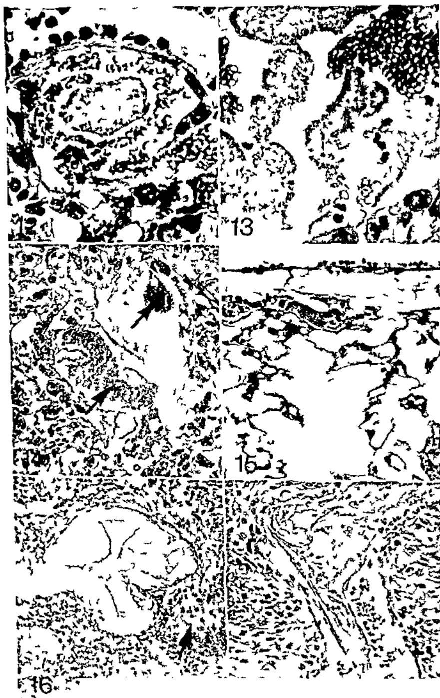

12
13
15
16

TABLE 2

Number of Mural Thrombi Predominantly Composed of Fibrin in the Lacunae of the "Roof"

Injection given	No of animals	Time of sacrifice after injection	Mean number of thrombi	S E.§
Untreated	10	–	6.3	± 0.73
Ellagic acid	10	3 min	5.[illegible]	± 0.55
Ellagic acid	10	10 min	5.1	± 0.46
Buffered saline	5	3 min	4.8	± 0.94
Buffered saline	5	10 min	6.3	± 1.75
Buffered saline	5	24 hrs	5.7	± 0.60

Mean of means per mother animal
§ Standard error of the mean

Fig. 5 also shows the relative numbers of placentas with only dense platelet aggregates without fibrin in the lacunae and in the uterine veins. Dense platelet aggregates that were found together with platelet fibrin masses are not expressed in the figure. When the recorded values for only dense platelet aggregates are considered separately no systematic trend of variation among the experimental groups is seen.

Neither did the frequency of placentas with mural fibrin thrombi show significant differences among the groups of animals studied (Table 2).

Table 3 shows that in the tubular area there was an unexplained high number of dense platelet aggregates in untreated animals. There was no significant difference in the number of dense platelet aggregates after injection of buffered saline or ellagic acid. No platelet fibrin masses or mural fibrin thrombi were seen in this area.

Thus the main difference between the controls and the ellagic acid treated animals killed after 3 and 10 minutes is in the frequency of platelet fibrin masses in the lacunae and the uterine veins.

TABLE 3

Number of Dense Platelet Aggregates Observed in Tubular Area

Injection given	No of animals	Time of sacrifice after injection	Mean number of platelet aggr	S E §
Untreated	10	–	8.0	± 1.45
Ellagic acid	10	3 min	4.2	± 1.55
Ellagic acid	10	10 min	3.6	± 1.10
Buffered saline	5	3 min	6.9	± 2.38
Buffered saline	5	10 min	5.6	± 1.61
Buffered saline	5	24 hrs	4	± 0.22

Mean of means per mother animal
§ Standard error of the mean

Sacrifice at 24 Hours after Injection of Ellagic Acid

In 74 out of 97 placentas the entire placenta was necrotic and 3 placentas showed partial necrosis

In the necrotic placentas (Figs 15 and 16) the number of dense platelet aggregates platelet fibrin masses and fibrin thrombi could not exactly be counted Therefore the group of animals killed 24 hours after ellagic acid injection are not included in Fig 5 and in Tables 2 and 3

The lacunae of the giant cell layer especially those centrally located were considerably dilated and filled with platelet fibrin masses erythrocytes and a few leucocytes (Fig 16) Blood cells had leaked into the surrounding areas of necrotic trophoblast and into the junctional zone between the giant cell layer and the decidua (Fig 16)

In some cases a partial separation of the placenta from the uterine wall was observed

The uterine veins were dilated and thrombi composed of platelets and fibrin filled the lumen (Fig 17) The amount of fibrin in the thrombi were greater than at 10 minutes after the injection of ellagic acid The endothelium underneath these thrombi was often lacking (Fig 17) The myometrium seemed to be well preserved but often a large number of erythrocytes were found between the smooth muscle cells adjacent to myometrial vessels which contained thrombi

In the cases of partial necrosis only the area around the central artery was affected The adjacent lacunae of the giant cell layer were dilated and contained erythrocytes and extensive platelet fibrin masses The peripheral lacunae of the giant cell layer were not occluded In the lacunae of the roof dense platelet aggregates and platelet fibrin masses were found

In 20 placentas necrotic areas were not observed Platelet fibrin masses were occasionally found in the lacunae of the roof and in the lacunae of the giant cell layer and the amount of fibrin was greater than in the animals killed 3 and 10 minutes after ellagic acid injection The number of platelet aggregates in the tubular area and mural fibrin thrombi in lacunae of the roof were approximately the same as in placentas from animals killed 3 and 10 minutes after ellagic acid injection and in animals injected with buffered saline The same referred to thrombi in the uterine veins

Sacrifice at 10 Minutes and 24 Hours after Injection of Heparin and Ellagic Acid

When heparin was given prior to the injection of ellagic acid the formation of new platelet fibrin masses was prevented and their number was about the same as in untreated animals The occurrence of dense platelet aggregates and fibrin thrombi was approximately

the same as in untreated animals and animals treated with buffered saline

Electron Microscopy of Placentas 10 Minutes after Injection of Ellagic Acid or Buffered Saline

After ellagic acid injection densely packed platelets were seen in the lacunae of the roof and in the lacunae of the giant cell layer (Fig 18) In the periphery of the aggregates the platelets had lost their organelles More centrally in the aggregates the platelets had retained most of their organelles but showed varying degrees of pseudopod formation Granular masses of moderate electron density and fibrils suggestive of fibrin were encountered between the platelets and especially in the peripheral areas Occasionally red blood cells and leucocytes were seen between the platelets

Some of the platelets in the periphery of the aggregates appeared to be in close contact with microvilli of the syncytium

In the placenta taken 10 minutes after the injection of buffered saline only separate platelets were found in the lacunae of the roof and of the giant cell layer

Thrombosis in the Lungs Heart Liver and Kidneys

Dense platelet aggregates were often encountered in the small pulmonary vessels (Fig 19) in all animals examined both pregnant and non pregnant

Pregnant Mice

Platelet fibrin masses or fibrin thrombi were never observed outside the placenta and uterus in untreated animals and in animals given buffered saline (Table 4) In some of the pregnant mice injected with ellagic acid platelet fibrin masses were found in the pulmonary vessels (Fig 20) and the right ventricle of the heart most frequently at

---

Figs 18–20

Fig 18 Electron micrograph of a lacuna of the giant cell layer ten minutes after injection of ellagic acid Aggregated platelets partly separated by granular masses and fibres suggestive of fibrin are filling the lumen In the periphery the platelets have lost their organelles and have decreased cytoplasmic electron density More centrally the platelets have retained their organelles although pseudopod formation is seen × 12 000

Fig 19 Untreated pregnant mouse Pulmonary venule with a platelet aggregate HPS × 500

Fig 20 24 hours after injection of ellagic acid Platelet fibrin mass in pulmonary vein HPS × 75


19


20

TABLE 4

The Number of Pregnant Animals in which Platelet Fibrin Masses were found in Lungs Heart Liver and Kidneys

Injection given	Time of sacrifice after injection	No of animals	Lungs	Heart	Liver	Kidneys
Ellagic acid	3 min	10	2	0	0	0
Ellagic acid	10 min	10	2	0	0	0
Ellagic acid	24 hrs	10	6	2	1	1
Untreated animals and animals treated with buffered saline		25	0	0	0	0
Ellagic acid and heparin		10	0	0	0	0

For subdivision of the group see Table 1

24 hours after the injection of ellagic acid (Table 4) Platelet fibrin masses were seen only in the kidney of one animal and in the liver of another animal

In the pregnant mice in which heparin was given before the injection of ellagic acid no platelet fibrin masses were observed outside uterus and placenta

Non Pregnant Mice

No platelet fibrin masses were seen in the organs of any of the 20 non pregnant mice that were untreated or injected with buffered saline In the 20 non pregnant animals injected with ellagic acid platelet fibrin masses were observed in the right ventricle of the heart of one animal sacrificed 10 minutes after the injection of ellagic acid No other platelet fibrin masses were observed in these animals in the heart lungs kidneys or liver

Bleeding Time and Clotting Time after Injection of Ellagic acid

Table 5 shows the effect of pregnancy and ellagic acid on bleeding and whole blood clotting times There was no significant difference in bleeding time between non pregnant and pregnant mice Nor did the injection of ellagic acid significantly alter bleeding time in either pregnant or non pregnant animals Whole blood clotting time was not significantly different in untreated non pregnant and pregnant animals The injection of ellagic acid significantly shortened whole blood clotting time in pregnant animals (Wilcoxon test pregnant untreated + pregnant buffer vs pregnant ellagic acid $p < 0.01$) In non pregnant animals the injection of ellagic acid also gave a reduced median whole blood clotting time but the difference is not statistically significant

TABLE 5

The Effect of Ellagic Acid on the Bleeding and Clotting Times in Non Pregnant and 16 Days Pregnant Mice

	No of animals	Bleeding time (secs)		Whole blood clotting time (secs)	
		Median	Range	Median	Range
Non pregnant Untreated	10	210	110->600	90	30–190
Non pregnant Buffered saline	10	145	120->600	52 5	35–260
Non pregnant Ellagic acid	10	145	90– 200	45	30–120
Pregnant Untreated	10	190	140->600	77 5	60–210
Pregnant Buffered saline	10	225	190->600	75	40–130
Pregnant Ellagic acid	10	170	120->600	45	30–120

TABLE 6

Fibrinogen Determination in Mice
The Individual Highest Dilution Titre with Precipitate

	No of animals	Titre		
		1/80	1/160	1/320
Non pregnant	10	1	9	0
Pregnant	10	0	8	2
Pregnant 10 minutes after ellagic acid	10	0	8	2
Pregnant 24 hours after ellagic acid	10	0	7	3

Fibrinogen Determination

ble 6 shows fibrinogen values in non pregnant and pregnant mice l in pregnant mice given ellagic acid There was no obvious dif ence between the groups The injection of ellagic acid did not result a fall of the fibrinogen level

DISCUSSION

ght microscopy of the mouse placenta showed at day 16 of gesta n platelet aggregates platelet fibrin masses and fibrin thrombi in e maternal blood channels Thus as in the normal human placenta 7) there is a tendency to thrombosis in the mouse placenta

Dense platelet aggregates were found in nearly all areas of the aternal blood channels in the placenta and in the uterine veins the brin thrombi were especially located to the lacunae and the uterine ins

Occasionally non occluding dense platelet aggregates in the lacunae and in the uterine veins appeared to be in contact with the vascular lining These aggregates could represent the initial stage in the development of fibrin thrombi (10 13) Platelet fibrin masses in contact with the vascular lining and fibrin mural thrombi with and *without persisting identifiable platelet masses are probably the later* stages The same stages in the development of fibrin thrombi could be recognized in the human placenta (17) Some of the mural thrombi were entirely or partly composed of a structureless or finely granular material In the previous study of the human placenta evidence was presented which indicated that these hyaline deposits were composed of aged fibrin (16 17)

Most of the dense platelet aggregates in the lacunae and in the uterine veins appeared to be floating free These aggregates could be emboli from platelet aggregates attached to upstream placental structures they could be cross sections of pendulating mural platelet aggregates or they could have been formed in flowing blood (10) as in Chandler s loop (4) Considering the small number of aggregates in contact with the vascular lining it is likely that a great proportion of them are formed in the flowing blood

Even if the dense platelet aggregates are formed in flowing blood they may secondarily give rise to mural platelet thrombi and later to mural fibrin thrombi *Jorgensen et al* (12) showed that platelet aggregation in the microcirculation may give rise to focal vascular damage and mural thrombi The morphological evidence of vascular damage included rupture of the peripheral membrane of the endothelial cells discharge of the cellular organelles and later disappearance of the cells underneath the mural thrombi Also in the present study the lining cells underlying the mural dense platelet aggregates and platelet fibrin masses occasionally showed signs of alterations Underneath the mural fibrin thrombi the lining cells were often lacking These changes could well be the result of a secondary focal injury in association with primary platelet aggregation in flowing blood On the other hand the signs of damage could also be a morphological expression of a primary focal alteration in the vascular lining leading to mural thrombosis However altered lining cells not associated with thrombotic materials were rarely seen

In a previous paper (17) we summarized observations which gave strong evidence for the view that platelet massing in vivo is largely independent of the exposed surface and is rather governed by the flow pattern From the structure of the human placenta we deduced that marked irregularities in flow were bound to occur (17) Similar reflections can be done in the case of the mouse placenta When *the blood enters the lacunae of the roof from* the narrow central artery the flow must be split and the direction abruptly changed This will necessarily entail the formation of a lot of eddies In the tubular

area the flow probably resumes a more linear pattern whereas irregularities of the flow may again occur in the wide lacunae of the giant cell layer and in the uterine veins

Mural fibrin thrombi were found in the areas with assumed flow irregularities i e in the lacunae and in the uterine veins In the lacunae of the roof the thrombi were particularly frequent in curved parts or small pockets where eddy formation is bound to be marked In the tubular area where the flow may be more linear no distinct fibrin thrombi were seen The dense platelet aggregates seen in this part of the placenta could be emboli from the platelet masses found in the lacunae of the roof

An increased local stimulation of the coagulation through release of tissue thromboplastin could accelerate thrombus formation In the human placenta and decidua (23) the content of tissue thromboplastin is very high However the external coagulation system plays a minor role for the stabilization of platelet aggregates (9 11)

In conclusion dense platelet aggregates and mural thrombi form in the maternal blood spaces of the normal mouse placenta as in the human placenta It is likely that disturbances of flow are important for the formation of the thrombotic material Alterations or disappearance of lining cells underneath some of the mural thrombi may be a phenomenon secondary to the thrombosis Release of tissue thromboplastin locally could accelerate the thrombus formation but is probably of minor importance

Increased coagulation activity of the maternal blood in general is *also* considered to promote thrombus formation in the normal human placenta (for review see 17) However the relatively crude method for fibrinogen determination (7 24) presently employed did not reveal *obvious* differences between pregnant and non pregnant mice and the whole blood clotting times were similar in the two groups Platelet fibrin masses or fibrin thrombi were not observed in organs outside *placenta* in animals not given ellagic acid Thus by these parameters evidence of a generalized increased tendency towards thrombosis could not be found in the pregnant mouse

A local and/or generalized diminished fibrinolytic activity may also play a role suggested by the persistence of fibrin in the placental thrombi and the lack of morphological signs of lysis *Phillips et al* (19) *injected* thrombin into pregnant and non pregnant rats and found that thrombi tended to remain longer in renal glomeruli in *pregnant* animals compared with non pregnant this may be due to diminished fibrinolytic activity during gestation

Like thrombi in the human placenta (17) the thrombi in the mouse *placenta* did not undergo organization in the usual way Leucocytes were rarely attracted by the thrombi in the placenta in contrast to thrombi in the uterine veins where leucocytes were often seen within them Occasionally the *syncytium* partly or totally covered

the surface of the fibrin thrombi analogous to the endothelialization of the thrombi in the uterine vessels In the lacunae of the giant cell layer and the uterine veins where the lining cells underneath thrombi were lacking fibrin in the thrombi occasionally appeared to merge with similar masses in between the underlying cells This could be due to diffusion of plasma into the surrounding tissue Another possibility is that the cells have proliferated into the base of the thrombus *i e* it represents an attempt of organization

Ellagic acid injected intravenously into pregnant mice shortened the whole blood clotting time This is due to an activation of the Hageman factor (20) and thus the intrinsic coagulation system However the whole blood clotting time in non pregnant animals was not significantly accelerated after injection of ellagic acid A shortening of the whole blood clotting time after injection of ellagic acid is found in non pregnant animals in other species (2 5) The bleeding time was not significantly altered after injection of ellagic acid However previous authors have found that injection of ellagic acid causes shortening of the bleeding time (5 6 18)

In the present study intravenous injection of ellagic acid resulted within few minutes in a considerable increase of platelet fibrin masses in the maternal blood channels of the placenta To a smaller extent platelet fibrin masses appeared even in the lungs At 24 hours after the injection of ellagic acid most of the placentas contained increased amounts of thrombotic material At that time the lungs of the pregnant mice contained more platelet fibrin masses than shortly after the injection occasionally at 24 hours such masses were seen in the heart liver and kidneys as well In non pregnant mice given ellagic acid platelet fibrin masses were only seen in the heart in one animal This is in agreement with *Nordoy & Chandler* (18) who did not find platelet fibrin masses in the organs of male rats 3 minutes after the injection of comparable doses of ellagic acid Only when ellagic acid has been combined with injection of ADP or Sephadex particles (18) or with stagnation of flow (2) have platelet fibrin masses been obtained These observations suggest that ellagic acid does not induce thrombosis unless another thrombogenic factor is present as well Therefore the high incidence of platelet fibrin masses in the maternal vascular channels of the placenta after the injection of ellagic acid confirms that there is a particular propensity within the placenta for thrombosis Since platelet fibrin masses were found in smaller number in other organs as well in ellagic acid treated pregnant mice it seems reasonable to assume that the pregnant state in mice entails a certain generalized tendency towards thrombosis However this generalized trend most likely plays a minor role for the formation of placental thrombi compared to the local thrombogenic factors

Most of the platelet fibrin masses seen 3 minutes after the ellagic

acid injection were found in the uterine veins and in the lacunae particularly in the lacunae of the roof. At 10 minutes a further increase in the size of platelet fibrin masses were found in the lacunae of the roof. Spontaneously occurring platelet fibrin masses and mural fibrin thrombi had the same predilection sites as the platelet fibrin masses found after ellagic acid injection. Thus the ellagic acid appears to accelerate the thrombogenic mechanism which already operates spontaneously in the placenta. As already discussed the most important factor for the propensity to thrombosis in the predilection sites may be disturbances of flow and this factor may be the additional stimulus necessary for ellagic acid to induce the increased amounts of platelet fibrin masses.

The fibrin formation after injection of ellagic acid was only observed in association with platelet aggregates and one must assume that the platelet fibrin masses have been preceded by platelet aggregates without fibrin. Already within 3 minutes enough thrombin must have formed to cause swelling and degranulation of the platelets (8) and to convert fibrinogen to fibrin. It cannot be decided whether the increased amount of platelet fibrin masses are mainly caused by more frequent stabilization of steadily formed reversible platelet aggregates (11) or whether more aggregates are formed by the extensive release of ADP from platelets under the influence of thrombin (14). The stimulation of the coagulation process by ellagic acid was completely prevented by heparin administration prior to the ellagic acid.

Three and 10 minutes after the injection of ellagic acid the number of mural fibrin thrombi in the lacunae of the roof was approximately the same as in animals treated with buffered saline and in untreated animals. Thus mural fibrin thrombi are not produced by ellagic acid within 10 minutes. Twenty four hours after ellagic acid injection most of the placentas showed necrotic changes. This group is therefore not suitable for a quantitative study of the transformation of thrombi induced by ellagic acid. In the well preserved myometrium however some thrombi with an appearance corresponding to about 24 hours age were found in the veins. The thrombi seemed to be attached to the vessel wall and the endothelium underneath the thrombi was often lacking. These findings could be explained by assuming that the ellagic acid not only gives rise to free floating platelet fibrin masses but also to fibrin rich stable thrombi apparently attached to the wall of the vascular channels. *Jorgensen et al* (12) showed that mural thrombi may form secondarily to platelet aggregation in flowing blood. It therefore seems possible that stable thrombi formed during the 24 hours after the injection of ellagic acid are secondary to increased platelet massing in the maternal channels shortly after the injection.

In conclusion injection of ellagic acid appears to highly accelerate the spontaneous thrombosis within the maternal blood channels of

the mouse placenta At the same time only a small number of platelet fibrin masses appear in the lungs and very few in the other organs examined This supports the concept that the placenta is an organ with particular tendency towards thrombosis The acceleration of the thrombotic process is probably brought about by a more rapid stabilization of reversible platelet aggregates or more extensive release of ADP from the platelets under the influence of thrombin The increased coagulation activity induced by ellagic acid took place only in association with platelet aggregates which perhaps primarily are formed as a result of disturbances of flow

SUMMARY

The occurrence of thrombi in the normal labyrinthine placenta of the 16 days pregnant mouse was investigated

Densely packed platelet aggregates were seen in nearly all parts of the maternal blood channels of the placenta and in the myometrial veins Platelet fibrin masses and mural fibrin thrombi were found in the lacunae of the roof and of the giant cell layer as well as in the myometrial veins

There was no invasion of leucocytes into the thrombi of the placenta and signs of organization were not observed Clear morphological evidence of fibrinolysis was lacking In the uterine veins however leucocytes were often observed within the thrombi

Thrombotic material was not observed in the lungs heart liver or kidneys

The intravenous injection of ellagic acid resulted in greatly increased amounts of platelet fibrin masses exclusively located to the lacunae of the roof and of the giant cell layer as well as to myometrial veins In most instances necrosis of the placentas was found 24 hours after the injection of ellagic acid No deaths occurred among the pregnant mice although platelet fibrin masses in the lungs and in the right ventricle of the heart could be seen at autopsy With one exception only thrombi were not observed in non pregnant mice treated with ellagic acid

In conclusion the mouse placenta is an organ with particular tendency towards thrombosis The injection of ellagic acid appears to accelerate the placental thrombosis A particular flow pattern may be the main stimulus for the formation of the thrombi The influence of increased concentration of coagulation factors and decreased fibrinolytic activity during pregnancy probably play a minor role

REFERENCES

1 *Amoroso E C* Placentation in the mouse and rat In Parkes A S (Ed) Marshall s Physiology of Reproduction vol 2 Longmans Green & Co., London New York Toronto 1952 p 258–270

2 *Botti R E & Ratnoff O D* Studies on the pathogenesis of thrombosis an experimental hypercoagulable state induced by the intravenous injection of ellagic acid J Lab clin Med 64 385-398 1964
3 *Caulfield J B* Effects of varying the vehicle for OsO in tissue fixation J Biophys Biochem Cytol 3 827-829 1957
4 *Chandler A B* In vitro thrombotic coagulation of the blood A method for producing a thrombus Lab Invest 7 110-114 1958
5 *Clifton E E* The effects of ellagic acid on the coagulation mechanisms and on traumatic bleeding Am J med Sci 254 483-490 1967
6 *Clifton E E Agostino D & Girolami A* Prevention of traumatic bleeding by ellagic acid in rats Proc Soc Exp Biol Med 120 179-180 1965
7 *Hjort P* Akutt fibrinogen mangel i svangerskapet Tidsskr for Den norske lægefor 76 756-760 1956
8 *Hovig T* The ultrastructure of rabbit blood platelet aggregates Thrombos Diathes haemorrh 8 455-471 1962
9 *Hovig T Rowsell H C Dodds W J Jørgensen L & Mustard J F* Experimental hemostasis in normal dogs and dogs with congenital disorders of blood coagulation Blood 30 636-669 1967
10 *Jørgensen L* Experimental platelet and coagulation thrombi A histological study of arterial and venous thrombi of varying age in untreated and heparinized rabbits Acta path microbiol scand 62 189-223 1964
11 *Jørgensen L & Borchgrevink C F* The haemostatic mechanism in patients with haemorrhagic diseases A histological study of wounds made for primary and secondary bleeding time tests Acta path microbiol scand 60 55-82 1964
12 *Jørgensen L Hovig T Rowsell H C & Mustard J F* Adenosine diphosphate induced platelet aggregation and vascular injury in swine and rabbits In preparation 1969
13 *Jørgensen L Rowsell H C Hovig T & Mustard J F* Resolution and organization of platelet rich mural thrombi in carotid arteries of swine Am J Path 51 681-719 1967
14 *Käser Clanmann R & Lüscher F F* The mechanism of platelet aggregation in relation to hemostasis Thrombos Diathes haemorrh 7 480-490 1962
15 *Lendrum A C Fraser D S Slidders W & Henderson R* Studies on the character and staining of fibrin J clin Path 15 401-413 1962
16 *Moe N* Deposits of fibrin and plasma proteins in the normal human placenta An immunofluorescence study Acta path microbiol scand 76 74-88 1969
17 *Moe N & Jørgensen L* Fibrin deposits on the syncytium of the normal human placenta evidence of their thrombogenic origin Acta path microbiol scand 72 519-541 1968
18 *Nordøy A & Chandler A B* Formation of platelet fibrin thrombi by ellagic acid and adenosine diphosphate in the rat Lab Invest 16 3-12 1967
19 *Phillips L L Margaretten W & McKay D G* Changes in the fibrinolytic enzyme system following intravascular coagulation induced by thrombin and endotoxin Am J Obst Gyn 100 319-326 1968
20 *Ratnoff O D & Crum J D* Activation of Hageman factor by solutions of ellagic acid J Lab clin Med 63 359-377 1964
21 *Reynolds E S* The use of lead citrate at high pH as an electron opaque stain in electron microscopy J Cell Biol 17 208-212 1963
22 *Romeis B* Mikroskopische Technik 15 ed Leibnitz Verlag München 1948 p 168
23 *Schneider C L* Thromboplastin complications of late pregnancy In Toxæmias of Pregnancy A CIBA Foundation Symposium J & A Churchill Ltd London 1950 p 163-181
24 *Schneider C L* Rapid estimation of plasma fibrinogen concentration and its use as a guide to therapy of intravascular defibrination Am J Obst Gyn 64 141-147 1952
25 *Skjørten F* Bilateral renal cortical necrosis and the generalized Shwartzman reaction I Review of literature and report of seven cases Acta path microbiol scand 61 394-404 1964
26 *Snedecor G W* Statistical Methods 5 ed The Iowa State University Press Ames Iowa 1956 p 115-121

Acta path microbiol scand 77 676–684 1969

From Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt Head Professor dr med S Dick Henriksen Oslo Norway

A SELECTIVE METHOD FOR THE ISOLATION OF *HAEMOPHILUS* IN MATERIAL FROM THE RESPIRATORY TRACT

By

BERIT HOVIG and EINAR H AANDAHL

Received 13 v 69

The bacteriology of the respiratory tract in infectious diseases has been the subject of numerous investigations (18 20) The varying results indicate that difficulties are encountered in these examinations and particularly in evaluating the significance of the different bacteria in cases of chronic bronchitis This is mostly due to the fact that the microbes commonly considered responsible for infections in the respiratory tract also inhabit the upper respiratory tract as commensals in healthy individuals

The lower respiratory tract (*i e* below larynx) has been found to be sterile in healthy individuals (1 12) and the diagnosis of infection of the lower respiratory tract is reliable only when based on examinations of bronchial secretion (4 16) Usually however one has to work on sputum samples where the bronchial secretion is mixed with the flora in the upper respiratory tract

Our routine method blood agar plates with a staphylococcus streak has been the method most commonly used in diagnostic work on respiratory tract samples As *Haemophilus influenzae* is one of the microbes most frequently connected with infection in the respiratory tract and as the diagnosis of this microbe is complicated by the abundant growth of other bacteria present other selective methods have been developed (6 18) All strains of *Haemophilus* are reported to be resistant to high concentrations of bacitracin (2 5) and thus bacitracin deserved trial as a selective substance

Other methods employed to facilitate the diagnostic work has been washing of the sputum samples to eliminate the usual oral flora and homogenization and liquefaction to obtain equal distribution of the microbes in the samples (13 15)

In this work we have examined the frequency of *Haemophilus* in cultures from nose and throat swabs and of sputum The routine blood

Requests for reprints should be addressed to Dr *B Hovig* Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt Rikshospitalet Oslo Norway

agar method has been compared to other selective methods using bacitracin as a selective agent Furthermore the effect of washing and homogenization in a smaller number of sputum samples on the isolation rate of various microbes has been tried out

MATERIALS AND METHODS

The present investigation has been carried out with nose and throat swabs and sputum samples of patients in this hospital with infections of the respiratory tract as well as with various other diseases

The routine method was direct plating of the samples on bovine or human blood agar plates streaked with a staphylococcus across the plates and examination for the satellite phenom non Purulent parts of the sputum samples were picked by the loop when plating The plates were incubated at 37 C overnight

Usually we have not distinguished between *Haemophilus influenzae* and *Haemophilus parainfluenzae* Where examination of X and V factor requirements was done X and V discs supplied by Bakteriologiska Laboratoriet Karolinska Sjukhuset Stockholm Sweden on Bacto Brain Heart Infusion Agar (Difco) medium were used

Washing and Homogenizing Procedure of Sputum (17)

Following inspection and Gram staining the samples were plated as described above Subsequently the sample was mixed thoroughly with four volumes of sterile saline and centrifuged at 3000 rpm for 10 minutes The supernatant was discarded and the deposit resuspended in an equal volume of a 1 per cent solution of pancreatic trypsin (supplied by Novo Terapeutisk Laboratorium A/S Copenhagen Denmark) The mixture was then shaken by hand for 5 minutes and placed in a water bath at 37 C for 60 minutes with occasional shaking prior to plating by the routine method

Selective Technique for the Identification of Haemophilus

The medium used was heated equine blood agar chocolate agar and bacitracin was used as the selective agent Two methods have been tried

1 Paper discs containing 10 IU bacitracin placed on the agar surface immediately after plating of the sample These discs were identical to those utilized for routine sensitivity tests and were supplied by Bakteriologiska Laboratoriet Karolinska Sjukhuset Stockholm Sweden Colonies appearing within the inhibition zone were picked and streaked on blood agar plates with a staphylococcus streak for the determination of satellitism If necessary one or more subcultures and Gram staining as well, were performed to ensure the diagnosis of *Haemophilus*

2 Incorporation of bacitracin in the chocolate agar medium Bacitracin was supplied by Apothekernes Laboratorium for Specialpræparater A/S Oslo Norway and contained 63 IU per mg Tests were performed to decide the highest concentration of bacitracin permitting optimal growth of all strains of *Haemophilus* 42 recently isolated strain were tested on medium with bacitracin concentrations ranging from 100 μg/ml to 2000 μg/ml Concentrations of 300 μg/ml or lower permitted full growth of all strains

A number of strains of other microbes frequently found in the specimens were also tested Strains of *Candida* sp *Escherichia* sp *Klebsiella* sp *Proteus* sp *Pseudomonas* sp and a few *Neisseria* were able to grow regardless of the concentration of bacitracin in the medium where as other microbes (e g *Diplococcus pneumoniae Streptococcus* sp *Staphylococcus* sp and *Micrococcus* sp) did not grow at all in medium with 240 μg/ml bacitracin or more

In the first part of this work a concentration of bacitracin of 240 μg/ml medium was used, but later this was increased to 300 μg/ml to inhibit more *Neisseria* strains Colonies appearing on the plates were subjected to the same procedure as described for the disc method In the comparative investigations the same loopful has been plated on both plates and care has been taken to deposit approximately the same amount of material

RESULTS

Using the routine method only we have examined the frequency of potentially pathogenic bacteria in 191 sputum samples from patients with chronic bronchitis (Table 1) Such microbes were found in 7 3 per cent of the samples but *Haemophilus* was only isolated from one sample

TABLE 1

Bacteriological Examination of Sputum Samples from Patients with Chronic Bronchitis Blood Agar Plates with Staphylococcus Streak

Total no of samples	Total no of samples with potentially pathogenic microbes
191	14

Staph aureus 3
Group A haemolytic streptococci 4 *Dipl pneumoniae* 1
Group A haemolytic streptococci & *Dipl pneumoniae* 2
Haemophilus 1

Table 2 gives the results of investigating the value of pretreating the sputum samples *i e* washing and liquefaction compared to the routine method only This elaborate technique does not represent any advantage neither as regards apathogenic nor potentially pathogenic bacteria In some samples *Neisseria* sp and *Streptococcus viridans* disappeared while at the same time Gram negative rods and *Staphylococcus aureus* appeared more abundantly It is worth noticing that *Haemophilus* was not isolated from any of these samples at all

Table 3 shows the comparison of two selective methods The use of chocolate agar with bacitracin incorporated in the medium was a more

TABLE 2

Bacteriological Examination of 30 Randomly Selected Sputum Samples Before and after Washing and Homogenization by Pancreatic Trypsin Blood Agar Plates with Staphylococcus Streak

Microbe isolated	No of samples	
	Before homogenization	After homogenization
Str viridans	22	19
Neisseria sp	[illegible]	13
Diphteroids	1	2
Dipl pneumoniae	1	1
Staph aureus	4	4
Pseudomonas	1	1
Enterobacteria	8	8

Other microbes supposed to belong to the normal flora *Staph epidermidis* *Micrococcus* sp non haemolytic streptococci and *Candida* sp displayed no difference before and after treatment with pancreatic trypsin

TABLE 3

Comparative Examination of two Selective Methods for the Isolation of Haemophilus *in Samples from the Respiratory Tract*

Total no of samples		No of samples with *Haemophilus*			
		Chocolate agar with bacitracin paper disc	Chocolate agar with 240 µg/ml bacitracin	Both methods	Total
Sputum	29	6	15	5	16
Throat	12	0	6	0	6
Throat§	54	15	35	14	36 (66 %)

Direct plating of randomly selected samples

§ Secondary culture on the two bacitracin media from primary blood agar cultures from patients in the oto rhino laryngological department with various diseases—*Haemophilus* was detected in only 5 (9 per cent) of the primary blood agar cultures

efficient method for the isolation of *Haemophilus* than the disc method and was the method adopted for further studies

The frequency of *Haemophilus* in 54 throat cultures from patients admitted to the oto rhino laryngological department is shown in the same table Secondary plating from blood agar plates (the loop picking material from the vicinity of the staphylococcus streak) to the selective media was used However three days were required to verify the presence of the bacterium using the bacitracin method this way and a quantitative estimation was impossible The isolation rate of

TABLE 4

Isolation Rate of Haemophilus *in Primary Cultures of Nose Throat and Sputum Samples*

Total no of samples		No of samples with *Haemophilus*	
		Blood agar plates with staphylococcus streak	Chocolate agar plates with bacitracin (240 and 300 µg/ml medium)
Nose	75	24 (32 %)	31 (41 3 %)
Throat	75	23 (30 7 %)	74 (98 7 %)§
Throat†	300	15 (5 %)	183 (61 %)
Sputum†	350	12 (3 4 %)	215 (61 4 %)

From patients with symptoms of infection in the upper respiratory tract

§ Of these strains 35 were *Haemophilus influenzae* and 20 strains were *Haemophilus parainfluenzae* whereas 19 strains were not identifiable with the technique used

† Randomly selected samples from hospitalized patients

Haemophilus in these samples rose from 9 to 66 per cent using the selective media

Simultaneous plating of the material on blood agar and selective medium makes the final bacteriological answer available in two days The results of a comparison between the two methods on the isolation rate are recorded in Table 4 The routine method revealed *Haemophilus* in a small number of samples in the unselected material whereas primary plating of the material on the bacitracin medium gave a high isolation rate of this microbe The 98 7 per cent isolation rate among patients with symptoms of upper respiratory tract infection is worth noticing Changing the concentration of bacitracin from 240 µg/ml to 300 µg/ml made no difference in the isolation rate of *Haemophilus* In two samples heavy seemingly pure growth of pneumococci was found with the routine method On selective plates however abundant growth of Haemophilus was obtained with the same material

DISCUSSION

The role of *Haemophilus influenzae* as a pathogenic microbe in the respiratory tract has been a controversial subject since its discovery in 1892 It has been considered responsible for a number of infectious diseases in the respiratory tract in addition to the classical influenza One of the reasons for this is that Koch s postulates have not been sufficiently kept in mind as criteria of pathogenicity The isolation of a certain bacterium from an open focus of infection is not necessarily sufficient reason to assume an etiological relationship Probably one has not fully realized the existence of *Haemophilus influenzae* in individuals without infectious diseases in the respiratory tract However the high prevalence of *Haemophilus* in normal individuals is a fact realized many years ago (7)

The evaluation of the clinical significance of *Haemophilus* in chronic bronchial diseases must be studied by means of bronchoscopy (1 4) A high frequency of *Haemophilus* in bronchial aspirates taken through the bronchoscope was found By a special swabbing device it is possible *to avoid contamination due to material being* carried with the bronchoscope from the upper respiratory tract to the lower (10 12 16) Using this technique the view of *Haemophilus* as an important agent in chronic bronchial disease is supported However it is apparent that cultures of sputum frequently do not provide a reliable picture of the bronchial flora When accurate bacterial identification is important bronchoscopy for obtaining uncontaminated samples is indicated Where such specimens are not available one has to use sputum or throat samples for diagnostic work

In patiens with chronic bronchitis a great number of published data on the bacteriological examination of sputum are available (18 *20)* English authors in particular have shown that in their country *Hae*

mophilus influenzae is predominantly connected with chronic bronchitis when examining sputum with a technique similar to our routine method (13 14 19). The isolation rates showed a variation between 20 and 55 per cent. Comparing these investigations to our results (Table 1) a strikingly low frequency of pathogenic bacteria in sputum from patients with chronic bronchitis was found. As most authors do not methodologically distinguish between *Haemophilus influenzae* and *Haemophilus parainfluenzae* in spite of their using the former term our results should be comparable as regards this. Allowing for geographical differences it nevertheless seems that the routine method described in our hands is unsuitable for the demonstration of the real frequency of *Haemophilus* in material from these sources.

The washing and liquefaction procedure of sputum samples (Table 2) did not increase the isolation rate of potentially pathogenic bacteria. This is in accordance with the results of *Lees & McNaught* (12). We found this technique laborious and cumbersome for routine use.

The demonstration of *Haemophilus* by means of the satellite phenomenon is an old method familiar to all bacteriologists. However the abundant flora in the upper respiratory tract considerably complicates the identification. These other microbes are usually less fastidious and grow more rapidly than *Haemophilus* the colonies of which are small and very easily overlooked and consequently they are almost impossible to obtain in pure culture. The possibility of an interaction or antagonistic effect on *Haemophilus* by other microbes or substances in sputum should be considered. Pneumococci are reported to suppress the growth of *Haemophilus* (13 20) and *Lapinski* (11) suggests some inhibitory factors to be present in sputum indicating this to be a combination of *Neisseria catarrhalis* and mucin. It seems that diagnosing *Haemophilus* in respiratory tract samples is more dependent on the other microbes present in the sample than on the actual organism itself.

Other selective methods are needed trying to find a selective agent which inhibits the growth of as many as possible bacteria in the sample except the haemophili of which 100 per cent of the strains encountered should be able to develop colonies. Probably *Fleming* (6) was one of the first to try out such a substance as he incorporated his penicillin in the medium to suppress the growth of bacteria other than *Haemophilus*. Incorporation of crystal violet has also been used (18).

Finland & Wilcox (5) investigated the action of antibiotics to 16 strains of *Haemophilus influenzae* and found all the strains to be resistant to 750 µg bacitracin per ml stating bacitracin to be essentially inactive against *Haemophilus influenzae*. The minimum inhibiting concentration (MIC) ranged from 750 to 3000 µg/ml.

Love & Finland (2) in a similar study of 90 strains of *Haemophilus influenzae* confirmed this. All strains grew in the presence of 1000 µg/ml bacitracin. MIC for penicillin in both studies were 12.5 µg/ml or

less for all the strains Due to this and to a better physico chemical stability than penicillin we decided to try bacitracin as a selective agent

It was evident (Table 3) that the incorporation of bacitracin in chocolate agar medium was better and more efficient than the disc method and we found that 300 μ_{D} bacitracin per ml medium was a satisfactory concentration However *Candida* sp and enterobacteria will grow at this concentration and apathogenic *Neisseria* will occasionally develop colonies An immediate plating of the samples on this medium will ensure the diagnosis of *Haemophilus* within 48 hours whereas secondary spreading from blood plates requires 72 hours before the final diagnosis being made

When making a comparison of the routine method and the bacitracin method is recorded in Table 4 it was evident that the isolation rate of *Haemophilus* in throat and sputum samples will be far too low if judged by the routine method only This did not to the same extent apply to nose cultures perhaps due to the normal flora in the nose usually being less abundant than in other parts of the upper respiratory tract and thus interfering less with the growth of *Haemophilus* A contributing fact may be that organisms which directly promote the growth of *Haemophilus* (*Staphylococcus* sp *Micrococcus* sp) are more commonly present in the nose than in throat and sputum Cultures of nose swabs in cases of infection such as sinusitis frequently give predominating growth of *Haemophilus*, often in enormous number thus making selective media superfluous

The question arises whether the high score of *Haemophilus* in the respiratory tract demonstrated by the bacitracin method represents clinically useful information or may lead to unnecessary antibacterial treatment directed against bacteria which are actually irrelevant to the disease of the patient The high score probably reflects the high incidence of *Haemophilus* in the normal flora in addition to the incidence of actual pathogens

We did observe that the growth on the bacitracin plates varied considerably from scanty to moderate or heavy growth We think that this semiquantitative estimation may bear correlation to the actual quantity in the sample but furher evaluation on this point is needed However it is interesting that heavy growth could occur on the bacitracin plate without satellite colonies being identified by the routine method and that the number isolated by the routine method does not necessarily parallel the quantity in the sample found by the bacitracin method As *Haemophilus* is the microbe most frequently connected with bronchial disease it seems logical to eliminate the unreliability of the sputum examinations due to unfavourable growth conditions for this microbe

It was stressed by *Henriksen* (8) that a real bronchial infection will become apparent by the pathogenic bacteria dominating the culture

even though the material is mixed with and contaminated by the flora of the pharynx and the oral cavity *Dixon & Miller* (3) based their dilution technique of sputum culture on the same hypothesis that an organism wich is causing inflammation of the lungs or bronchi will be present in sputum in considerably greater numbers than will organisms that have superficially contaminated the sputum during its passage through the pharynx and mouth *Kilbourn et al* (9) developed a quantitative method for bacteriological examination of sputum and stated that their lower limits of sensitivity were such that in liberating great number of organisms from sputum the few contaminating bacteria resulting from expectoration either were not found in colony counts or were of a low order of magnitude They used chocolate agar for the isolation of *Haemophilus* species

To judge whether bacterial counts of material from the respiratory tract in fact permit the separation of contamination from infection it is now necessary to make quantitative bacteriological examinations of throat sputum and bronchial secretion from healthy individuals as well as from patients with symptoms of infection in the respiratory tract We think that the use of the bacitracin medium must be included in such quantitative studies

In the present state of knowledge it seems that a heavy growth of *Haemophilus* on the bacitracin plates as well as clear clinical signs of infection must be demanded if an infection caused by the organism is to be assumed

SUMMARY

The frequency of *Haemophilus* has been studied in cultures from nose throat and sputum of patients with different respiratory diseases as well as patients with various other diseases

The authors have compared the use of blood agar plates with a staphylococcus streak with some other selective techniques for the isolation of *Haemophilus* The method adopted was incorporation of bacitracin in chocolate agar plates (300 $\mu g/ml$)

The isolation rate of *Haemophilus* in the samples was greatly increased and it is concluded that in order to reveal the real frequency of these microbes it is necessary to use a selective method However the necessity of correlating the bacteriological data with clinical information is stressed

REFERENCES

1 *Brumfitt W* W *Willoughby M L N & Bromley L* An evaluation of sputum examination in chronic bronchitis Lancet (*II*) 1305–1309 1957

2 *Del Love B & Finland M* Susceptibility of recently isolated strains of *Haemophilus influenzae* to eleven antibiotics in vitro J Pediat 45 531–537 1954

3 *Dixon J M S & Miller D C* Value of dilute inocula in cultural examination of sputum Lancet (*II*) 1046–1048 1965

4 *Edlund G Richtner N G Tunevall G & Wassermann J* The significance of *Haemophilus influenzae* in chronic respiratory disease as elucidated by serologic tests Acta oto laryng (Stockh) 52 316–320 1960
5 *Finland M & Wilcox C* In vitro susceptibility of *Haemophilus influenzae* to seven antibiotics Amer J clin Path 20 335–340 1950
6 *Fleming A* On the antibacterial action of cultures of a penicillium with special reference to their use in the isolation of *B influenzae* Brit J exp Path 10 226–236 1929
7 *Fleming A & MacLean I H* On the occurrence of influenza bacilli in the mouths of normal people Brit J exp Path 11 127–134 1930
8 *Henriksen S D* Studies on the bacterial flora of the respiratory tract Skr norske Vidensk Akad Mat nat Kl no 11 1–241 1936
9 *Kilbourn J P Campbell R A Grach J L & Willis M D* Quantitative bacteriology of sputum Amer Rev resp Dis 98 810–818 1968
10 *Kortekangas A E* Investigations of the bacterial flora of the respiratory tract Acta oto laryng (Stockh) 50 (suppl 150) 1–169 1959
11 *Lapinski E M Flakas E D & Taylor B C* An evaluation of some methods for culturing sputum from patients with chronic bronchitis and emphysema Amer Rev resp Dis 89 760–763 1964
12 *Lees A W & McNaught W* Bacteriology of lower respiratory tract secretions sputum and upper respiratory tract secretions in normals and chronic bronchitis Lancet (*II*) 1112–1119 1959
13 *May J R* Pathogenic bacteria in chronic bronchitis Lancet (*II*) 839–842 1954
14 *May J R & May D S* Bacteriology of sputum in chronic bronchitis Tubercle (Lond) 44 162–173 1963
15 *Mulder J* Bacteriology of bronchitis Proc roy Soc Med 49 773–776 1956
16 *Potter R T Rotman F Fernandez F McNeill T M & Chamberlain J M* The bacteriology of the lower respiratory tract Bronchoscopic study of 100 clinical cases Amer Rev resp Dis 97 1051–1061 1968
17 *Rawlins G A* Liquefaction of sputum for bacteriological examination Lancet (*II*) 538–539 1953
18 *Turk D C & May J R* *Haemophilus influenzae* Its clinical importance The English Universities Press Ltd London 140 p 1967
19 *Wade O L Elmes P C & Bartley E* Pathogenic organisms in the sputum of patients with chronic bronchitis Thorax 22 265–270 1967
20 *Zinnemann K* *Haemophilus influenzae* and its pathogenicity Ergebn Mikrobiol 33 307–368 1960—61

Acta path microbiol scand 77 685–697 1969

The Institute of Clinical Bacteriology (Head S Winblad M D)
University of Lund General Hospital Malmo Sweden

STUDIES ON *YERSINIA ENTEROCOLITICA*
GROWTH ON VARIOUS SOLID MEDIA AT 37° C AND 25° C

By

BIRGITTA NILÉHN

Received 16 v 69

In recent years *Yersinia enterocolitica* (*Schleifstein & Coleman*, 1943) *Frederiksen* 1964 has been isolated with increasing frequency from human intestinal contents (*Mollaret et al* 1964 *Wauters & Mollaret* 1965 *Winblad et al* 1966 a b *Graux & Wauters* 1966 *Nilehn* 1967 a b *Nilehn & Sjostrom* 1967 *Nilehn et al* 1968 *Van Noyen & Vandepitte* 1968 *Mal ulu et al* 1968) Various media used for the diagnosis of conventional enteric pathogens have proved useful for the isolation of the bacterium e g McConkey agar Desoxycholate citrate agar or modifications of it and SS agar *Nilehn & Sjostrom* (1967) who used LSU agar (*Juhlin & Ericson* 1961) succeeded in isolating *Yersinia enterocolitica* from enteric contents with a much higher frequency on incubation at 22 C than at 37 C Though the growth of a number of *Yersinia enterocolitica* strains mainly of animal origin has been studied in pure culture on different solid media (*Struve* 1963) few investigations have been made to find out the methods most suitable for demonstrating the bacterium in a mixed faecal flora

The purpose of the present investigation was to examine the growth of various *Yersinia enterocolitica* strains and above all the variants usually encountered in human beings on 4 different selective media otherwise used in the isolation of enteric pathogens Bacterial growth was studied at 37 C and 25 C with comparative colony counts and with isolation essays from a mixed faecal bacterial flora

MATERIAL AND METHODS

Bacterial Strains

Thirty five strains of *Yersinia enterocolitica* most of which were recent isolates of human origin were used in the tests In one case recent isolates of the same strain (MY 29a and MY 29b) on different media were used Those strains from other laboratories that were included in the investigation *were selected* so as to represent strains of various origins and different variants that have been referred to the *Yersinia enterocolitica group* These strains have often been described in the

The study was aided by *grants from the Medical Faculty of the University of* Lund Sweden and from the Swedish Medical ...

strains of human origin were diluted in saline so as to give about 10 colonies per plate when 0.1 ml volumes were spread on the various media as described above. Colony diameters were determined microscopically on S form colonies with a Leitz Messoccular × 10.

The isolation of Yersinia enterocolitica from a mixed faecal bacterial flora. Yersinia enterocolitica suspensions. Three ten fold serial dilutions in saline were prepared for each strain.

Faecal suspensions. Routine faecal samples (different for each *Yersinia enterocolitica* strain) were diluted 1 : 1000 (w/v) in saline.

The number of bacteria in the *Yersinia enterocolitica* dilutions as well as in the faecal dilutions were approximately estimated by viable counts on HB agar incubated aerobically at 37° C for 48 hr. One ml of each *Yersinia enterocolitica* dilution was then thoroughly mixed with each 1 ml of faecal suspension. The dilution factors of *Yersinia enterocolitica* in the mixed suspensions were designated 1, 10 and 100. The approximate numbers of colony forming units (cfu) in the final mixture (*Yersinia enterocolitica* dilution factor = 1) are given in Table 4. The mixed suspensions were streak cultured with a platinum loop yielding about 0.03 ml volumes on the 5 different media; duplicate plates were made for each dilution, medium and temperature. The plates were finally inspected after 2 days incubation at 37° C and 25° C respectively. Suspected *Yersinia enterocolitica* colonies were isolated and verified biochemically and serologically (*Nilehn* 1967a). The greatest dilution of *Yersinia enterocolitica* that permitted the isolation of the organism was registered. The amount of mixed faecal bacteria was crudely estimated and recorded as follows: +++ = heavy growth, ++ = moderate growth, + = scanty growth, — = no growth.

RESULTS

Comparative Colony Counts

Twenty *Yersinia enterocolitica* strains of various origin were tested with comparative colony counts at 37° C and 25° C on the 4 differential selective media and by the use of human blood agar as non inhibitory reference medium. The ability of the various media to support growth judged from the number of easily recognizable colonies was statistically evaluated by means of variance analysis, the results of which are given in Tables 1 and 2. The probability of differences in the table between colony counts on different media or at different temperatures being due to chance was less than 0.01.

All of the strains tested gave a maximum number of colonies on HB agar, usually without significant differences between plates incubated at 37° C and 25° C. For 2 strains (MY 123 and MY 99) however, the colony counts were somewhat lower at 37° C; in one case (MY 123) the bacteria also showed a marked rough dissociation at the higher temperature.

As to the growth on the 4 selective differentiating media, strains of human origin belonging to the hitherto commonest serotypes 3 and 9 (*Winblad* 1967, 1968, *Nilehn* 1967b) formed a fairly uniform group (Table 1). With the exception of strain Hässig 3/24 they showed only moderate and in 3 cases no significant decrease in the number of colonies on the 4 media studied compared with the results obtained on the reference medium. The degree of inhibition by different media is apparent from the confidence intervals of differences between colony counts on the reference medium and those on other media.

TABLE 1

Comparative Colony Counts (cfu) of 10 Yersinia enterocolitica Strains of Human Origin O Antigen Types 3 and 9 (Winblad 1967 1968) Tested on 5 Different Solid Media at 37 C and 25 C

ains	Mean cfu (n = 5) arranged according to size (1–10) 1	2	3	4	5	6	7	8	9	10	Confidence intervals on the level = 0 98 of differences in cfu between reference medium (1) and other media (2–10)
g 3/24	B_{25} 232	B_{37} 214	E_{25} 174	D_{37} 157	S_{25} 156	D_{25} 150	L_{25} 130	L_{37} 0	S_{37} 0	E_{37} 0	1– 3 (15–108) 1– 7 (57–154)
blad 0)	B_{37} 164	B_{25} 164	E_{37} 143	S_{37} 137	D_{37} 135	S_{25} 134	L_{37} 130	F_{25} 130	L_{25} 126	D_{25} 12	
57c	B_{37} 309	B_{25} 300	L_{25} 288	E_{25} 256	E_{37} 248	D_{25} 243	L_{37} 232	D_{37} 227	S_{25} 222	S_{37} 221	1– 10 (24–160)
37a	B_{37} 76	B_{25} 76	E_{25} 76	E_{37} 71	L_{37} 64	D_{37} 63	S_{25} 60	L_{25} 60	S_{37} 57	D_{25} 54	
107a	B_{37} 165	B_{25} 161	S_{37} 140	E_{37} 135	D_{37} 134	L_{37} 132	S_{25} 132	D_{25} 132	E_{25} 128	L_{25} 126	
112	B_{37} 260	B_{25} 255	E_{37} 189	D_{37} 188	L_{25} 187	L_{37} 186	E_{25} 185	S_{37} 182	D_{25} 161	S_{25} 152	1– 10 (53–166)
120	B_{37} 127	B_{25} 106	E_{25} 93	L_{37} 88	E_{37} 87	L_{25} 86	S_{37} 86	S_{25} 85	D_{37} 83	D_{25} 80	1– 10 (20– 81)
123	B_{25} [illegible]	B_{37} 221r	L_{25} 118	E_{25} 95	S_{37} 93	S_{25} 91	D_{25} 84	D_{37} 81	E_{37} 77	L_{37} 76	1– 2 (5–111) 1– 6 (128–248) 1– 10 (142–264)
39	B_{37} 275	B_{25} 253	E_{25} 223	E_{37} 208	S_{37} 198	S_{25} 196	D_{25} 195	D_{37} 187	L_{37} 187	L_{25} 179	1– 4 (2–151) 1– 10 (20–180)
79b	B_{25} 287	B_{37} 274	E_{37} 194	L_{37} 186	D_{37} 178	E_{25} 178	L_{25} 174	S_{25} 171	D_{25} 171	S_{25} 162	1– 10 (61–202)

Counts connected by underlining do not differ significantly ($P<0.01$) from one another

B = *Human blood agar (Nilehn & Sjostrdm 1967)* *L* = LSU agar (*Juhlin & Ericson* 1961) S = SS agar (Bacto) D = Desoxycholate citrate agar (Bacto) E = Endo agar (Bacto) r = rough dissociation

B_{37} L_{37} S_{37} D_{37} E_{37} = Media incubated at 37 C (48 hr)
B_{25} L_{25} S_{25} D_{25} E_{25} = Media incubated at 25 C (48 hr)

The greatest inhibition observed was that which corresponded to on the average a growth reduction to about 25 per cent of the growth on the reference medium (strain MY 123) As a rule in this group of strains no differences between cfu on the different selective differentiating media were found and no systematically better yield at either of the incubation temperatures The strain Hassig 3/24 dif

TABLE 2

Compaartive Colony Counts (cfu) of 10 Yersinia enterocolitica *Strains of Mixed Origins O Antigen Types 1–3 5 7 8 (Winblad 1967) Tested on 5 Different Solid Media at 37 C and 25 C*

Strains	Mean cfu (n = 5) arranged according to size (1–10) 1	2	3	4	5	6	7	8	9	10	Confidence intervals on level = 0 98 of differences cfu between reference medi (1) and other media (2 10)		
Becht 200 (T 356)	B_{37} 239r	B_{25} 233	E_{25} 133	D_{25} 131	D_{37} 129	S_{25} 125	L_{25} 82	L_{37} 72r	S_{37} 0	E_{37} 0	1– 6 (83–147)	1– 8 (134–202)	
Dickinson 280 1 (P 254)	B_{37} 143	B_{25} 137	E_{37} 134	D_{37} 105	E_{25} 101r	S_{37} 80r	L_{37} 56r	D_{25} 28	L_{25} 28	S_{25} 26	1– 6 (25–109)	1– 7 (48–137)	1– (73 1
Becht 51 (T 348)	B_{37} 166	B_{25} 154	E_{25} 137	F_{37} 133	L_{37} 117	S_{25} 115r	S_{37} 110	D_{37} 80	D_{25} 65r	L_{25} 4	1– 7 (11– 97)	1– 9 (60–162)	
Daniels 975 (T 335)	B_{37} 130r	B_{25} 110r	E_{25} 104	E_{37} 98	S_{37} 66	L_{37} 63	S_{25} 46r	D_{37} 35	D_{25} 23r	L_{25} 0	1– 4 (2– 69)	1– 7 (50–124)	1– ([illegible]
Daniels 1028 P 377	B_{37} 216	B_{25} 207	L_{25} 153	E_{25} 149	E_{37} 143	D_{25} 91	D_{37} 75	L_{37} 75	S_{37} 14	S_{25} 0 2	1– 5 (33–119)	1– 8 (98–191)	
Lucas 405 (P 369)	B_{37} 269	B_{25} 244	L_{37} 0	L_{25} 0	S_{37} 0	S_{25} 0	D_{37} 0	D_{25} 0	F_{37} 0	E_{25} 0			
Albany 33114 (P 310)	B_{37} 281	B_{25} 267	E_{37} 179	E_{25} 174	L_{25} 68	L_{37} 64	S_{25} 62	D_{25} 61	S_{37} 60r	D_{37} 59	1– 4 (55–167)	1– 10 (162–289)	
M Y 99	B_{25} 395	B_{37} 341	E_{25} 341	L_{25} 313	E_{37} 311	L_{37} 219	D_{25} 167	D_{37} 122	S_{25} 5	S_{37} 0	1– 5 (31–142)	1– 6 (121–238)	1 (1,0-
Vache (Ye 123)	B_{37} 198	B_{25} 178	E_{37} 163	E_{25} 159	L_{37} 159	L_{25} 158	D_{25} 114	D_{37} 104	S_{37} 0	S_{25} 0	1– 6 (6– 79)	1– 8 (58–130)	
Jument (Ye 124)	B_{25} 151	B_{37} 134	L_{37} 123	E_{25} 121	L_{25} 113	L_{37} 109	D_{25} 68	D_{37} 1	S_{37} 0	S_{25} 0	1– 6 (10– 89)	1– 7 (48–127)	

Counts connected by underlining do not differ significantly (P<0 01) from one another

B = Human blood agar (Vilén *& Sjöström* 1967) L = LSU agar (*Juhlin & Ericson* 1961) S = SS agar (Bacto) D = Desoxycholate citrate agar (Bacto) E = Endo agar (Bacto) r = rough dissociation

B_{37} L_{37} S_{37} D_{37} E_{37} = Media incubated at 37 C (48 hr) (Strains Daniels 1028 and Lucas 405 72 hr)

B_{25} L_{25} S_{25} D_{25} E_{25} = Media incubated at 25 C (48 hr) (Strains Daniels 1028 and Lucas 405 72 hr)

fered however from the other human strains of the same O antigen type in that it grew poorly at 37 C on LSU agar SS agar as well as on Endo agar

The group of strains taken together in Table 2 which were het

erogeneous regarding origin and properties showed relatively large differences in growth with the medium used and often also with the incubation temperature The strain Becht 200 isolated from dog showed like the human strain Hassig 3/24 inhibition of growth at 37 C on SS agar and Endo agar and despite a certain growth strong rough dissociation on LSU agar at this temperature One strain (Lucas 405) isolated from hare gave no growth within 72 hours on any of the selective media studied at either temperature Among the remaining 8 strains 4 were practically completely inhibited on SS agar at both temperatures LSU agar inhibited growth of 2 chinchilla strains strongly on incubation at 25 C DC agar gave total inhibition in only one case (strain Jument) and at 37° C but rather low colony counts at both temperatures in 6 cases On the average the highest counts were noted in the case of Endo agar without any substantial difference between incubation at 37 C and 25 C

Colonial appearence of Yersinia enterocolitica

The morphology of the colonies on the different media agreed largely with that described in previous publications (*Struve* 1963 *Wauters & Mollaret* 1965 *Winblad et al* 1966b *Nilehn & Sjostrom* 1967)

After 24 hours the growth of *Yersinia enterocolitica* strains recently isolated from human specimens was hardly visible on LSU agar at either temperature on the other media tested the colony size varied from pinpoint to at most 1 mm in diameter S form colonies were somewhat larger at 37 C than at 25 C but a certain tendency towards rough dissociation was sometimes observed at the higher temperature even on the non inhibitory reference medium Table 3 shows the range of variation of the diameters of the S form colonies on the respective media at 37 C and 25 C after 48 hours incubation LSU agar gave the smallest colonies while the size did not vary appreciably with the other media tested

Among strains of animal origin the colonial size varied substantially

TABLE 3

Diameter of S Form Colonies of Yersinia enterocolitica *on Human Blood Agar LSU Agar (Juhlin & Ericson 1961) Bacto SS Agar Bacto Desoxycholate Citrate (DC) Agar and Bacto Endo Aga after 48 hr Incubation at 37 C and 25 C Ten Strains Recently Isolated from Human Specimens (O Antigen Type 3 of Winblad 1967) Measured at an Inoculation Density of about 10 cfu/Plate (Petri dishes 9 cm in Diameter 28 ml Medium/Dish)*

Diam of colonies (mm)	Human blood agar		LSU agar		SS agar		DC agar		Endo agar	
	37 C	25 C	37 C	25 C	37 C	25 C	37 C	25 C	37 C	25 C
mean	2.1	1.9	1.6	1.2	2.0	1.8	1.8	1.6	2.1	1.8
range	1.4–2.9	1.2–2.3	1.0–2.0	1.0–1.6	1.0–2.9	1.2–2.3	1.4–2.2	1.1–2.1	1.2–2.9	1.1–2.0

TABLE 2

Compaartive Colony Counts (cfu) of 10 Yersinia enterocolitica *Strains of Mixed Origins O Antigen Types 1–3 5 7 8 (Winblad 1967) Tested on 5 Different Solid Media at 37 C and 25 C*

Strains	Mean cfu (n = 5) arranged according to size (1–10) 1	2	3	4	5	6	7	8	9	10	Confidence intervals on level = 0 98 of differences cfu between reference medi (1) and other media (2 10)		
Becht 200	B_{37}	B_{25}	E_{25}	D_{25}	D_{37}	S_{25}	L_{25}	L_{37}	S_{37}	E_{37}	1– 6	1– 8	
(T 356)	239r	233	133	131	129	125	82	72r	0	0	(83–147)	(134–?0?)	
Dickinson 280 1	B_{37}	B_{25}	E_{37}	D_{37}	L_{25}	S_{37}	L_{37}	D_{25}	L_{25}	S_{25}	1– 6	1– 7	1
(P 254)	143	137	134	105	101r	80r	56r	28	28	?6	(25–109)	(48–137)	(73 1
Becht 51	B_{37}	B_{25}	E_{25}	E_{37}	L_{37}	S_{25}	S_{37}	D_{37}	D_{25}	L_{25}	1– 7	1– 9	
(T 348)	166	154	137	133	117	115r	110	80	65r	4	(11– 97)	(60–16?)	
Daniels 975	B_{37}	B_{25}	E_{25}	E_{37}	S_{37}	L_{37}	S_{25}	D_{37}	D_{25}	L_{25}	1– 4	1– 7	1
(T 335)	130r	110r	104	98	66	63	46r	35	23r	0	(?– 69)	(50–124)	(
Daniels 1028	B_{37}	B_{25}	L_{25}	E_{25}	E_{37}	D_{25}	D_{37}	L_{37}	S_{37}	S_{25}	1– 5	1– 8	
P 377	216	207	153	149	143	91	75	75	14	0 2	(33–119)	(98–191)	
Lucas 405	B_{37}	B_{25}	L_{37}	L_{25}	S_{37}	S_{25}	D_{37}	D_{25}	E_{37}	E_{25}			
(P 369)	269	244	0	0	0	0	0	0	0	0			
Albany 33114	B_{37}	B_{25}	E_{37}	E_{25}	L_{25}	L_{37}	S_{25}	D_{25}	S_{37}	D_{37}	1– 4	1– 10	
(I 310)	281	?67	179	174	68	64	62	61	60r	59	(55–167)	(16?–?89)	
MY 99	B_{25}	B_{37}	E_{25}	L_{25}	L_{37}	L_{37}	D_{25}	D_{37}	S_{25}	S_{37}	1– 5	1– 6	1–
	395	341	341	313	311	219	167	1?2	5	0	(31–142)	(1?1–?38)	(170–
Vache	B_{37}	B_{25}	E_{37}	E_{25}	L_{37}	L_{25}	D_{25}	D_{37}	S_{37}	S_{25}	1– 6	1– 8	
(Ye 123)	198	178	163	159	159	158	114	104	0	0	(6– 79)	(58–130)	
Jument	B_{25}	B_{37}	E_{37}	E_{25}	L_{25}	L_{37}	D_{25}	D_{37}	S_{37}	S_{25}	1– 6	1– 7	
(Ye 1?4)	1?1	134	123	121	113	109	68	1	0	0	(10– 8?)	(48–127)	

Counts connected by underlining do not differ significantly ($P<0\ 01$) from one another

B = Human blood agar (Viléhn & Sjostrom 1967) L = LSU agar (*Juhlin & Ericson* 1961) S = SS agar (Bacto) D = Desoxycholate citrate agar (Bacto) E = Endo agar (Bacto) r = rough dissociation

B_{37} L_{37} S_{37} D_{37} E_{37} = Media incubated at 37 C (48 hr) (Strains Daniels 1028 and Lucas 405 72 hr)

B_{25} L_{25} S_{25} D_{25} E_{25} = Media incubated at 25 C (48 hr) (Strains Daniels 1028 and Lucas 405 72 hr)

fered however from the other human strains of the same O antigen type in that it grew poorly at 37 C on LSU agar SS agar as well as on Endo agar

The group of strains taken together in Table 2 which were hel

Streak Cultures) Comparison of 5 Different Solid Media at 37 C and 25 C

unchanged number of faecal bacteria)
rowth of faecal bacteria§

SS agar		DC agar		Endo agar	
37 C	25 C	37 C	25 C	37 C	25 C
100/+	100/—	100/—	100/—	100/++	100/+
100/++	100/—	100/++	100/—	1/+++	1/+++
100/+	100/+	-/+++	100/+	-/+++	1/+++
1/++	10/++	1/++	10/++	-/+++	1/++
100/—	100/—	10/—	100/—	1/++	10/++
1/++	10/++	-/++	100/+	-/+++	-/++
100/++	100/+	1/++	100/+	1/+++	-/++
/++	100/—	-/+++	100/+	-/+++	1/++
10/++	100/+	10/++	100/++	-/+++	1/++
1/+++	10/++	-/+++	1/++	-/+++	-/+++
10/++	1/++	-/++	10/++	-/+++	-/+++
10/++	10/+	10/++	100/—	-/+++	1/++
1/++	-/++	1/+++	1/++	-/+++	-/+++
100/++	100/++	100/—	100/—	-/+++	-/+++
1/+++	100/+	1/+++	10/+++	-/+++	-/+++

of Yersinia in the specimens The possibility of isolating Yersinia usually varied with the degree of inhibition of Gram negative conta minants The poorest results were thus obtained on the non inhibitory HB agar and Endo agar without any appreciable difference between these media

It applied to all selective differentiating media that incubation at 25 C resulted in a higher degree of inhibition of disturbing Gram negative flora respectively a larger number of isolations of *Yersinia enterocolitica* than incubation at 37 C On incubation at 25 C no appreciable differences between the results obtained on LSU agar SS agar and DC agar were found while the possibility of isolating *Yersinia enterocolitica* at 37 C appeared to be somewhat less good on DC agar than on the other two media Strains or different isolates of the same strain primarily isolated at different temperatures or on different media showed no differences in growth that might suggest that adaptation to a certain medium or a certain temperature had occurred

DISCUSSION

The purpose of this investigations was above all to find suitable methods for isolating *Yersinia enterocolitica* from human intestinal

contents by primary plating Interest was therefore focused on such variants most often observed in human beings (*Nilehn* 1967a *Nilehn & Sjostrom* 1967 *Winblad* 1967 *Niléhn et al* 1968) For comparison however also some strains mostly of animal origin and belonging to other biochemical and/or serological groups were included in the trial

Four media otherwise used for the isolation of conventional enteric pathogens were tested for their ability to support growth of *Yersinia enterocolitica* in pure culture and in a mixed flora The ability of Bacto SS agar Bacto Desoxycholate citrate agar (DC agar) and Bacto Endo agar all commercially available (Difco) to differentiale and possibly to select ordinary intestinal pathogens from a human intestinal flora is well known *Juhlin & Ericson* (1961) have described selective and differentiating properties of LSU agar as to Salmonellae and Shigellae this medium has also recently given good results in the isolation of *Yersinia enterocolitica* from human intestinal contents (*Nilehn & Sjostrom* 1967 *Niléhn et al* 1968)

Strains of human origin (serotypes 3 and 9) usually gave relatively good growth on all media used at both 37 C and 25 C in some cases even without any significant decrease in the number of colonies on selective media compared with that obtained on the reference medium On comparison of the results obtained with strains primarily isolated on different media or at different temperatures no differences due to possible adaption to a certain medium or certain temperature were observed The occurrence of rough dissociation in some cases at 37 C agreed with earlier observations on newly isolated strains (*Nilehn & Sjostrom* 1967)

Older type strains of different origin and of different properties were found to differ appreciably from one another in degree of growth with the medium as well as with the incubation temperature used *Burrows & Gillett* (1966) found differences in nutritional requirements for growth among a small number of *Yersinia enterocolitica* strains and invariably greater demands on growth factors at 37 C than at 28 C So far however our knowledge of the nutritional requirements and differences in metabolic behaviour of this group of bacteria is not sufficient to allow theoretical considerations on differences in growth on different complex media The degree of adaptation or other changes of strains kept for a long time at different laboratories must of course also be regarded as uncertain

The variation of the tested strains suggest that difficulties are involved in the finding of a differentiating and preferably also selective medium suitable for the isolation of all biotypes of *Yersinia enterocolitica* At one or both temperatures Endo agar proved to be able to support a fairly good growth of all organisms included in the study with the exception of an extremely poorly growing strain (Lucas 405) biochemically deviating from most of the other variants studied

However as usual in attempts to isolate a pathogenic organism from a mixed faecal flora the ability of a medium to support growth of the enteric pathogen is not the only factor of importance it must also preferably suppress the growth of normal contaminants This applies especially as in this case to the search for slowly growing bacteria whose colonies even after two days incubation appear minute compared with those of ordinary heavily growing coliform bacteria As expected owing to the dominating Gram negative faecal flora above all swarming Proteus blood agar used as a non inhibitory reference medium often gave poor results in attempts to isolate *Yersinia enterocolitica* This applies also to Endo agar which hardly inhibited Gram negative bacteria in the faeces more than did the reference medium On the other three media tested the increased inhibition of undesired faecal bacteria on incubation at 25 C proved to be advantageous in the isolation of *Yersinia enterocolitica* of the common type from human faecal specimens These findings are also supported by previous experience in the isolation of the bacterium from human intestinal contents (*Nilehn & Sjostrom* 1967) A systematic comparison between incubation at 22 C and at 37 C then showed a much greater frequency of isolation of *Yersinia enterocolitica* at the low temperature by the use of LSU agar as primary medium and Rappaport (*Rappaport et al* 1956 *Rappaport & Konforti* 1959) broth as enrichment medium

A comparison between results of isolation on LSU agar SS agar and DC agar at 25 C does not suggest that any of these media is superior to the others *Yersinia enterocolitica* in low concentrations could usually be demonstrated on all The differentiating capacity which however is largely dependent on the subjective evaluation and experience of the examiner was perhaps somewhat better on LSU agar where *Yersinia enterocolitica* colonies assumed a characteristic appearance owing to an initial alcaline reaction probably due to urease activity Proteus colonies could usually be readily distinguished from Yersinia because of their size

The investigation thus suggest that *Yersinia enterocolitica* can be satisfactorily isolated from human faecal samples if suitable selective differentiating media are used such as LSU agar SS agar and DC agar Incubation at 25 C increases the possibility of isolating strains of the type most often encountered in human material

In attempts to isolate other variants it appears advisable however to use incubation at 37 C as well and if possible a combination of several different media which as in the search for other enteric pathogens presumably increases the number of positive cultures In view of the slow growth of the bacteria the incubation time should never be less than 2 days irrespective of the plating medium

SUMMARY

Four media conventionally used in the diagnosis of enteric pathogens Bacto SS agar Bacto Desoxycholate citrate agar Bacto Endo agar and LSU agar were investigated for their ability to support growth at 37 C and 25° C of *Yersinia enterocolitica* in pure culture and in culture from artificially infected faecal samples Strains isolated from human beings (serotype 3 and 9) usually showed good growth on all the media used at both temperatures except a certain tendency to rough dissociation at 37 C Older type strains of different origins and representing different biochemical and/or serological variants differed in growth with the medium used and with the incubation temperature

In isolation experiments on human strains of serotype 3 from artificially infected faecal samples incubation at 25 C gave better results than at 37 C probably because of the increased inhibition of faecal contaminants at the lower temperature On incubation at 25 C equally good results were obtained on SS agar DC agar and LSU agar

REFERENCES

1 *Burrows T W & Gillett W A* The nutritional requirements of some Pasteurella species J gen microbiol 45 333–345 1966

2 *Frederiksen W C* A study of some Yersinia pseudotuberculosis like bacteria (*Bacterium enterocoliticum* and *Pasteurella X*) Proc XIVth scand Congr Path Microbiol Oslo 103–104 1964

3 *Graux C & Wauters G* Infection familiale à *Yersinia enterocolitica* Acta clin Belg 21 206–215 1966

4 *Jacober F X* L infection à *Yersinia enterocolitica* une Zoonose d avenir (These) Copedith Paris 1968

5 *Juhlin I & Ericson C* A new medium for the bacteriologic examination of stools (LSU agar) Acta path microbiol scand 52 185–200 1961

6 *Makulu A Gatti F Mollaret H H & Vandepitte J* Human intestinal infections with *Yersinia enterocolitica* in Kinshasa VIIIth internat Congr on Tropical Medicine and Malaria Teheran 1–5 1968

7 *Miller R J Jr* Simultaneous statistical Interference Mc Graw Hill New York 1966

8 *Mollaret H H Chevalier A & Deplanche M C* Contribution à l étude d un noveau groupe de germe proches du bacille de Malassez et Vignal Ann Inst Pasteur 107 121–127 1964

9 *Mollaret H H & Lucas A* Sur les particularités biochimiques des souches de *Yersinia enterocolitica* isolées chez les lièvres Ann Inst Pasteur 108 121–125 1965

10 *Niléhn B* Studies on *Yersinia enterocolitica* Characterization of 28 strains from human and animal sources Acta path microbiol scand 69 83–91 1967

11 *Niléhn B* Bacteriological studies on 80 strains of *Yersinia enterocolitica* of human origin Internat Symp on Pseudotuberculosis Paris 1967 Symp Series immunobiol Standard Karger Basel/New York 9 327–336 1968

12 *Niléhn B & Sjostrom B* Studies on *Yersinia enterocolitica* Occurrence in various groups of acute abdominal disease Acta path microbiol scand 71 612–628 1967

13 *Niléhn B Sjöström B Damgaard K & Kindmark C* *Yersinia enterocolitica* in patients with symptoms of infectious disease Acta path microbiol scand 74 101–113 1968

14 *Niléhn B* Electron microscopic studies on flagellation in different strains of *Yersinia enterocolitica* Acta path microbiol scand 77 527–541 1969

15 *Rappaport F Konforti N & Navon B* A new enrichment medium for certain Salmonellae J clin Path *9* 261–266 1956

16 *Rappaport F & Konforti N* Selective medium for paratyphoid bacteria Applied Microbiol 7 63–66 1959

17 *Struve M* Untersuchungen mit Bakterienstammen vorlaufig als *Pasteurella X*" bezeichneten Bakterienart Inaug Diss Tübingen 1963

18 *Van Noyen R & Vandepitte J* L isolement de *Yersinia enterocolitica* par une technique usuelle de coproculture Ann Inst Pasteur *114* 463 467 1968

19 *Wauters G & Mollaret H H* Un noveau cas d infection humaine à *Yersinia enterocolitica* Rev Belge Path *31* 328–333 1965

20 *Winblad S Niléhn B & Jonsson M* Two further cases bacteriologically verified of human infection with *Pasteurella X* (Syn *Yersinia enterocolitica*) Acta path microbiol scand *67* 537–541 1966

21 *Winblad S Nilehn B & Sternby N H* *Yersinia enterocolitica (Pasteurella X)* in human enteric infections Brit med J *2* 1363–1366 1966

22 *Winblad S* Studies on serological typing of Yersinia enterocolitica The XVth scand Congr Path Microbiol Copenhagen 115–116 1967

23 *Winblad S* Personal communication 1968

Acta path microbiol scand 77 698–706 1969

The Department of Clinical Bacteriology University of Umeå Umeå Sweden and the Department of Microbiology University of Lund Lund Sweden

EFFECTS OF ENDOTOXIN ON TISSUE CULTURE CELLS

By

S BERGMAN and C WEIBULL

Received 22 v 69

Cytotoxicity of endotoxin *in vitro* has been demonstrated in various ways Thus *Heilman* (1963 1965 1968) observed morphological effects on organ explants *Kessel, Braun & Plescia* (1966) compared the increase in number of Trypan Blue positive macrophages which were washed and exposed to endotoxin with the corresponding number of macrophages maintained in a non toxic solution *Woods et al* (1961) showed that hydrolysed *Salmonella* endotoxin lost its lethality for mice parallel with its potency of stimulating glycolysis in tumour cells Effects of endotoxin of foetal chick fibroblasts were studied by *Bergman & Nilsson* (1963) An inhibition of the growth of the fibroblasts was noted

Assays for endotoxic activity involving animals or embryos are expensive and difficult to standardize With the inexpensive slide technique of *Bergman & Nilsson* (1963) a number of endotoxin preparations can be tested simultaneously under standardized conditions The aim of the present investigation was to study the system endotoxin fibroblasts further and to test the suitability of this system as a quantitative assay for endotoxin

MATERIALS AND METHODS

The following endotoxin preparations were used 1 A lipopolysaccharide extracted with phenol water (*Westphal Lüderitz & Bister* 1952) from cell walls of *Proteus mirabilis* strain VI (*Weibull et al* 1967) 2 Bacto Lipopolysaccharide *E coli* 055 B5 (Difco Laboratories Detroit USA) control number 594181 3 Bacto Lipopolysaccharide *S marcescens* (Difco) control number 521440 4 Bacto Lipopolysaccharide *S typhi murium* (Difco) control number 462074

The chick embryos were obtained from a white leghorn breed After the viscera and head of eight days old embryos were removed the embryos were carefully cut into small pieces The cells were suspended in Parker 199 medium supplemented with 20 mg of tryptose 100 μg of streptomycin and 100 I U of penicillin G per ml In addition calf serum was added to the growth medium at a final concentration of 2 per cent

This work was supported by research grant 69 6 from the Swedish Cancer Society

The cells were allowed to grow in Carrel flasks for four days the medium being renewed every day The cells were detached with crystalline trypsin (Trypure Novo) The enzyme was dissolved in a modified Hanks BSS solution (*Hanks* 1948 *Spång berg* 1969) and added to the cell medium at a final concentration of 0 01 per cent After washing with Hanks BSS the cells were counted and suspended in the same fluid at a final concentration of 600 000 cells per ml

HeLa cells and fibroblasts from human foetuses were suspended in Parker 199 medium supplemented with 2 per cent calf serum at final concentrations of 200 000 and 100 000 cells per ml respectively

The effects of endotoxin on the tissue culture cells were studied essentially as described previously (*Bergman* & *Nilsson* 1963) The endotoxins were dissolved in 0 85 per cent saline To each slide 0 2 ml of test solution and 0 5 ml of cell suspension were added The cells were allowed to attach to the slides for four hours at 37 C The rings were then removed and the slides were placed in Hellenthal cuvettes containing 40 ml of the nutrient medium After 18 hours the cells were fixed in Carnoy s fluid and stained with haematoxylin according to Weigert s method. Examination of the cells was made as described by *Bergman* (1963)

RESULTS

Table 1 shows the results of an experiment with 8 day old chick embryo cells exposed to various concentrations of endotoxin extracted with phenol water from cell walls of *Proteus mirabilis* strain VI (1 2) It can be seen that the endotoxin markedly inhibited the attachment of the embryo cells to glass slides and that the number of mitoses in the attached cells decreased with increasing toxin concentrations

To test more closely the dose response relationships in the system Proteus endotoxin chick embryo cells we carried out several experiments of the type illustrated in Table 1 Different cell preparations were used for each experiment The number of cells and mitoses

TABLE 1 a

Effect of Proteus Endotoxin on the Number of 8 Day Old Chick Embryo Cells Attaching to Glass Slides

Slid	Final concentration of endotoxin (µg/ml)					Saline
	0 57	0 29	0 11	0 057	0 029	
1	274	292	224	334	610	410
2	372	332	252	180	306	660
3	280	[illegible]8	442	350	416	340
4	304	300	186	410	354	642
5	198	370	462	306	300	482
6	200	404	452	350	472	400
7	140	252	430	432	428	464
8	104	414	346	378	468	300
9	110	372	320	394	400	370
10	[illegible]	270	352	482	260	476
Average values	219 ± 24	316 ± 23	347 ± 32	362 ± 26	401 ± 33	454 ± 39

Each slide culture was prepared from 0 5 ml of cell suspension and 0 2 ml of endotoxin solution Cell suspension (0 5 ml) mixed with 0 2 ml of saline served as controls The figures give the numbers of cells observed in 20 fields of vision The ± signs indicate standard errors

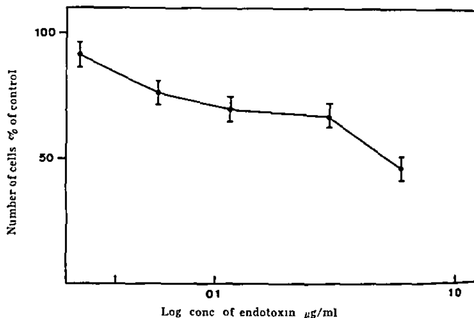


Fig 1 a

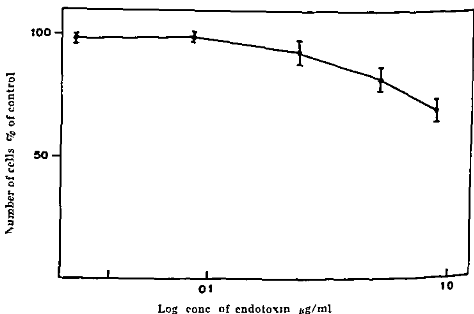


Fig 1 b

Relationship between number of 8 day old chick embryo cells attaching to glass slides and concentrations of endotoxin in the surrounding medium Relative values for the numbers were used the numbers found in the endotoxin free controls being put equal to 100 The vertical bars give standard deviations of the means a Proteus endotoxin Average values of data obtained from ten experiments are given b Serratia endotoxin Average values of data obtained from five experiments are given

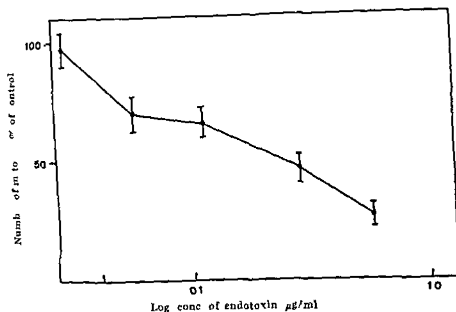


Fig 2 a

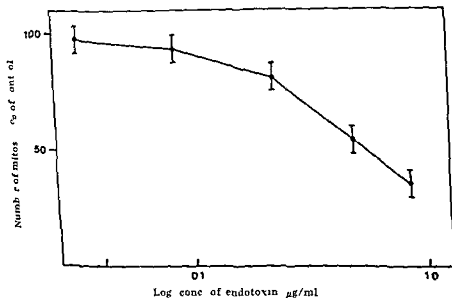


Fig 2 b

Relationship between number of mitoses in chick embryo cell cultures and concentration of endotoxin in the surrounding medium For details see legend of Fig 1
a Proteus endotoxin b Serratia endotoxin

TABLE 4

Number of HeLa Cells Attaching to Glass Slides and Number of Mitoses Occurring in these Cells

Slide	Final concentration of endotoxin (μg/ml) 2.9		1.1		0.57		0.29		Saline	
	Cells	Mitoses	Cells	Mitoses	Cells	Mitoses	Cells	Mitoses	Cells	Mitoses
1	176	5	194	6	190	7	196	5	186	13
2	–	–	220	2	204	4	194	3	218	7
3	202	5	198	11	202	5	218	2	198	10
4	206	2	202	2	202	3	206	4	204	8
5	200	9	192	5	210	10	192	0	182	6
6	202	3	188	1	190	4	222	5	182	2
7	206	4	182	5	200	0	178	7	186	6
8	–	–	208	7	186	4	–	–	222	6
9	218	10	188	1	190	4	184	3	240	6
10	200	3	202	0	208	2	192	1	186	4
Average values	191 ± 4	5.1 ± 1.0	197 ± 4	[illegible] ± 1.0	198 ± 3	4.3 ± 0.9	178 ± 5	3.3 ± 0.7	200 ± 6	6.8 ± 1.0

Each slide culture was prepared from 0.5 ml of cell suspension and 0.2 ml of Proteus endotoxin solution. Cell suspension (0.5 ml) mixed with 0.2 ml of saline served as controls. The figures give the number of cells or mitoses observed in 20 fields of vision. The ± signs indicate standard errors.

TABLE 5
Number of Fibroblasts from Human Foetuses Attaching to Glass Slides

Slide	Final concentration of endotoxin (μg/ml) 2.9	0.57	Saline
1	186	162	124
2	188	136	236
3	154	178	158
4	206	120	170
5	178	132	150
Average values	182 ± 8	146 ± 11	167 ± 19

Each slide culture was prepared from 0.5 ml of cell suspension and 0.2 ml of Proteus endotoxin solution. Cell suspension (0.5 ml) mixed with 0.2 ml of saline served as controls. The figures give the number of cells observed in 20 fields of vision. The ± signs indicate standard errors.

(*Heilman* 1967). Moreover, the fact that different endotoxins gave different dose response curves in our study indicates that our method can hardly be used for precise measurements of the relative activities of various toxins. However, this method is simple, rapid and inexpensive. Many tests can be carried out simultaneously. It may therefore be useful for semiquantitative estimations of toxic activities.

Several biological activities are probably involved in the various assays used for estimations of endotoxins. The dose response curves obtained by us indicate complex effects of the endotoxin on the chick embryo cells.

According to *Kessel, Braun & Plescia* (1961), cytotoxic effects of endotoxins *in vitro* seem to require the presence of endotoxin specific antibody at the surface of susceptible cells. However, these workers point out that a primary non-immunological toxicity may also exist. The results obtained by us favour the latter suggestion. It seems rather improbable that antibodies against endotoxins from four different bacterial genera (*Escherichia, Salmonella, Serratia, Proteus*) were present in our test systems. However, it is not easy to explain why the fibroblasts are especially sensitive to endotoxin at an age of 7 to 8 days. Also the non-sensitivity of HeLa cells and fibroblasts from human foetuses remains unexplained. The specificity of the reaction between the chick embryo cells and endotoxin is demonstrated by the fact that dextran and endotoxin hydrolysed with acid left these cells unaffected. On the other hand, treatment of the endotoxin with alkali did not influence the endotoxin cell reaction. In this connection it should be mentioned that *Neter et al.* (1956) found that such a treatment markedly increases the erythrocyte modifying capacity of bacterial endotoxins.

As has been shown by *Bladen, Gewurtz & Mergenhagen* (1967), the

complement factors C 3-C 9 of the guinea pig are involved in the biological action of endotoxin We plan to investigate this finding further with our test system

SUMMARY

The effect of various endotoxins (bacterial lipopolysaccharides) on chick embryo fibroblasts HeLa cells and fibroblasts from human foetuses were studied by means of a simple slide technique Only the chick embryo cells were sensitive to the toxins The technique used may be useful for semi quantitative estimations of toxic activities Probably a primary non immunological toxicity of the lipopolysaccharides caused the effects observed

REFERENCES

1 *Bergman S* Culture of human fibroblasts on glass plates Acta path microbiol scand *59* 279–291 1963
2 *Bergman S & Nilsson S B* Effect of endotoxin on embryonal chick fibroblasts cultured in monolayer Acta path microbiol scand *59* 161–164 1963
3 *Bladen A H Gewurt H & Mergenhagen S E* Interactions of the complement system with the surface and endotoxic lipopolysaccharide of Veillonella alcalescens J exp Med *125* 767–786 1967
4 *Finkelstein R A* Observations on mode of action of endotoxin in chick embryos Proc Soc exp Biol *115* 702–707 1964
5 *Hanks J H* The longevity of chick tissue cultures without renewal of medium J cell comp Physiol *31* 235–240 1948
6 *Heilman D H* Tissue culture methods for studying delayed allergy a review Tex Rep Biol Med *210* 136–157 1963
7 *Heilman D H* The selective toxicity of endotoxin for phagocytic cells of the reticuloendothelial system Int Arch Allergy *26* 63–79 1965
8 *Heilman D H & Bast R C Jr* In vitro assay of endotoxin by the inhibition of macrophage migration J Bact *93* 15–20 1967
9 *Heilman D H* In vitro toxicity of endotoxin for macrophages of young guinea pigs and rabbits Int Arch Allergy *33* 501–510 1968
10 *Kessel R W I Braun K & Plescia O J* Endotoxin cytotoxicity Role of cell associated antibody Proc Soc exp Biol *121* 449–452 1966
11 *Neter F Westphal O Lüderitz O Gorzynski E A & Eichenberger E* Studies of enterobacterial lipopolysaccharides Effects of heat and chemicals on erythrocyte modifying antigenic toxic and pyrogenic properties J Immunol *76* 377–385 1969
12 *Spangberg L* Biological effects of root canal filling materials Odont Revy *20* suppl 16 1969
13 *Weibull C Bickel W D Haskins W T Milner K C & Ribi E* Chemical biological and structural properties of stable *Proteus* L forms and their parent bacteria J Bact *93* 1143–1159 1967
14 *Westphal O Lüderitz O & Bister F* Ueber die Extraktion von Bakterien mit Phenol/Wasser Z Naturforsch *7b* 148–155 1952
15 *Woods M W Landy M Whitby J L & Burl D* Symposium on bacterial endotoxins Bact Rev *25* 447–456 1961

Acta path microbiol scand 77 707-716 1969

Laboratory of Oral Microbiology Department of Microbiology The Gade Institute and Institute of Periodontology Schools of Medicine and Dentistry University of Bergen Norway

IMMUNOCHEMICAL STUDIES OF ORAL FUSOBACTERIA

5 Serological Characterization of a Group Reactive Antigen

By

TORE KRISTOFFERSEN

Received 23 iv 69

In two earlier communications the purification and chemical properties of a group reactive precipitinogen found in strains of oral fusobacteria and termed Precipitinogen 2 have been described (8 9) The material contained protein a carbohydrate component consisting of glucose and xylose and small amounts of lipid The precipitating ability of the antigen has been shown to be destroyed or reduced by 45 per cent phenol by digestion with proteolytic enzymes (pronase and pepsin) and by periodate oxidation (7)

The present study describes some serological and antigenic properties of the isolated substance

MATERIALS AND METHODS

Precipitinogen 2 was isolated from *Fusobacterium* strain F1 as described for preparation no 1 in (9)

Rabbit immune sera against whole bacteria strain F1 were produced as described previously (7) The sera were inactivated at 56 C for 30 minutes

The techniques used for *ring test* and *agar precipitation tests* have been described (7)

For *complement fixation tests* two 100 per cent lytic units of guinea pig complement and two units of hemolysin were used A veronal buffer containing optimal concentrations of Ca and Mg was used as dilutent (6) Fixation of complement was allowed to take place at 4 C overnight A 1 per cent suspension of washed and packed (1600 × g 30 minutes) sheep erythrocytes in the proper concentration of hemolysin was allowed to stand at room temperature for 15 minutes and subsequently added to each test tube The tubes were then incubated at 37 C for 0 minutes and the degree of hemolysis was estimated visually The antigen and inactivated antiserum ere controlled not to possess anticomplementary or lytic properties

Indirect hemagglutination was attempted with

a) Sheep erythrocytes washed three times in phosphate buffered saline pH 7 2 (= NSE)

b) Sheep erythrocytes which had been treated with tannic acid (= TSE) To a 2 per cent suspension of washed and packed sheep erythrocytes in buffered saline

These investigations were supported by grants from *L Meltzer's Høyskolefond* and from the *Norwegian Research Council for Science and the Humanities*

was added an equal volume of tannic acid diluted 1 40 000 The suspension was left on the bench for 30 minutes washed three times with saline and finally suspended in buffered saline.

c) Formalinized sheep erythrocytes prepared by the method of *Daniel et al* (2) as described by *Milgrom et al* (10) The formalinized erythrocytes were treated with tannic acid diluted 1 20 000 as described above and suspended in saline prior to sensitization (= TFSE)

For sensitization of sheep erythrocytes a solution of the antigen in buffered saline was added to an equal volume of a 2 per cent suspension of sheep erythrocytes The mixture was incubated at 37 C for 30 minutes The sensitized erythrocytes were washed three times and finally suspended in buffered saline at a 1 per cent concentration For the reaction with TSE it proved necessary that the diluent contained 0 5 per cent normal inactivated rabbit serum as a stabilizer (5)

The sera to be tested were diluted 1 2 and absorbed with packed NSE. Two fold serial dilutions of test serum in 0 2 ml volumes were added to test tubes with 0 9 mm internal diameter With NSE and TSE 0 1 ml of 1 per cent sensitized erythrocytes was added to each test tube In experiments with TFSE 0 ? ml of the erythrocyte suspension was found to give good results The tubes were left on the bench for 1 hour incubated at 4 C overnight and again left on the bench for 30 minutes before reading the agglutination patterns

For *inhibition of indirect hemagglutination* 0 2 ml volumes of two fold serial dilutions of antigen in buffered saline were used and 8 agglutinating units of antiserum in 0 2 ml volumes were added to each tube The mixtures were incubated at 37 C for 1 hour and 0 2 ml of 1 per cent sensitized TFSF was added. Incubation and reading took place as described for the indirect hemagglutination test

Antiserum was fractionated on Sephadex G 200 (Pharmacia Uppsala Sweden) according to the method described by *Flodin & Killander* (3) The column used was 2 5 × 45 cm and 5 ml fractions were collected after automatic recording of the transmittance by use of the Uvicord I equipment (LKB Produkter AB Stockholm Sweden) The fractions were dialyzed against buffered saline and used in ring test precipitation and for indirect hemagglutination tests using TFSE as described.

For *reductive cleavage of antibodies* 2 mercaptoethanol (ME) was added to the serum or to fractions from the Sephadex G 200 column to a final concentration of 0 1 M The mixture was left on the bench for 1 hour and an equal volume of 0 15 M iodoacetamide was added After standing on the bench for another hour the mixture was dialyzed against buffered saline overnight at 4 C

Enzymatic digestions and periodate oxidation of the antigen Digestion with *pepsin* (2 × crystallized Sigma) took place in 0 0? M acetate buffer pH 4 1 The enzyme substrate ratio was varied from 1 50 to 1 12 5 (w w) After digestion the suspensions were neutralized by addition of 0 1 M phosphate buffer pH 7 4 For digestion with *β glucosidase* (ex almonds Koch Light) a 0 0? M acetate buffer pH 5 0 was used The enzyme substrate ratio was 1 2 5 to 1 10 in different experiments The digests were neutralized as described above Digestion with *maltase* (α glucosidase) (ex yeast Koch Light) took place in 0 05 M phosphate buffer pH 6 7 The enzyme substrate ratio was varied as described for β glucosidase Digestion with the different enzymes took place at 37 C for ?0 hours Within the limits of this study the effects for each of the enzymes were the same with the various enzyme substrate ratio used *Periodate oxidation* was carried out in 0 05 M Na *meta* periodate in 0 05 M phosphate buffer pH 7 2 and was allowed to proceed for 20 hours in the dark

Test solutions and controls could be dialyzed against buffered saline for up to 1 hour with gentle agitation inside and outside the dialysis bag More extensive dialysis was not feasible because of the instability of the antigen during such procedures (8)

In all the above experiments controls without enzyme or periodate were included In experiments in which the test solutions were used for inhibition of indirect hemagglutination with TFSE controls containing enzyme or periodate only were also tested The residual periodate or enzymatic activities were not found to influence the tests

EXPERIMENTS AND RESULTS

Precipitation

Precipitinogen 2 in concentrations up to 10 mg per ml gave one distinct line on agar precipitation with undiluted antiserum to whole bacteria F1 (wells 2 and 7 Fig 1) The line was visible with concentrations of the antigen as low as 30 µg per ml As reported in a previous paper (8) one band was formed in immunoelectrophoresis experiments With an 0 1 per cent solution of the antigen a potent antiserum gave a titre of 1 16 in agar precipitation tests

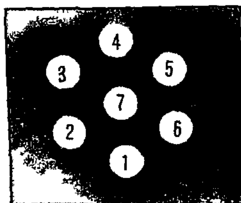


Fig 1

Agar double diffusion plate showing lines formed with purified Precipitinogen 2 and different homologous antisera The plate was photographed after 48 hours Wells were filled with

1 Rabbit antiserum to erythrocytes sensitized with purified antigen
3 Normal rabbit serum
5 Rabbit antiserum to purified antigen
7 Rabbit antiserum to whole microorganisms stra n F1
Wells Nos 2 and 6 contained purified antigen 0 1 mg per ml

The lowest concentration of Precipitinogen 2 which gave precipitation in the ring test was approximately 6 µg per ml Potent antisera gave a ring test titre of 1 64 with an 0 1 per cent dilution of the antigen

Complement Fixation

Different concentrations of Precipitinogen 2 were used in complement fixation tests with two fold serial dilutions of antiserum Table 1 gives the results of such an experiment Smaller amounts of antigen could be detected by complement fixation than in precipitation tests The highest serum titres 1 320 were obtained with concentrations from 6 25 to 50 µg per ml of antigen High concentrations of antigen slightly inhibited the reaction

TABLE 1

Fixation of Complement by Precipitinogen 2 and Antiserum Strain F1

Dilution of 0.1 per cent of antigen	Dilutions of homologous antiserum 1:20	1:40	1:80	1:160	1:320	1:640	1:1280
1:10	4	3	3	1	–	–	–
1:20	4	4	4	3	1	–	–
1:40	4	4	4	3	1	–	–
1:80	4	4	4	3	1	–	–
1:160	4	4	4	3	1	–	–
1:320	4	4	4	3	–	–	–
1:640	4	4	3	1	–	–	–
1:1280	4	4	3	–	–	–	–
1:2560	4	1	–	–	–	–	–
1:5120	–	–	–	–	–	–	–

1 to 4 Slight to complete inhibition of hemolysis — Complete hemolysis

Indirect Hemagglutination and Inhibition of Hemagglutination

Antisera failed to agglutinate NSE treated with different concentrations of Precipitinogen 2. Furthermore no hemolysis occurred when 0.2 ml of active guinea pig serum diluted 1:15 was added to the test tubes. Adsorption of Precipitinogen 2 to NSE therefore could not be demonstrated.

The antigen was readily adsorbed to tanned sheep erythrocytes. Good results were also obtained with erythrocytes which had been formalinized prior to treatment with tannic acid. No stabilizing agent was necessary with such cells and the results were very reproducible. Maximal titres were obtained when a solution containing 60 to 250 µg/ml of antigen was used for sensitization of an equal volume of 2 per cent TFSE in saline. For further experiments a 100 µg/ml solution of the antigen has been employed. The titres with different antisera against whole microorganisms strain F1 varied somewhat from 1:8 192 to 1:32 768, most sera giving a titre of 1:16 348.

Precipitinogen 2 in concentrations of 0.25 µg/ml completely inhibited the agglutination of sensitized TFSE when 8 agglutinating units of antiserum were used.

Antigenicity of Precipitinogen 2 and the Nature of Antibodies in Immune Serum

A solution containing 2 mg of Precipitinogen 2 in 0.5 ml of saline was injected intracutaneously and subcutaneously in a rabbit. After two weeks the rabbit was bled. The undiluted antiserum produced a precipitation line in agar with Precipitinogen 2. This line gave a "reaction of identity" with the line seen with antiserum to whole bacteria but was less sharp and not so well defined as the latter (Fig. 1, wells 5, 6 and 7). In the indirect hemagglutination test with TFSE the titre

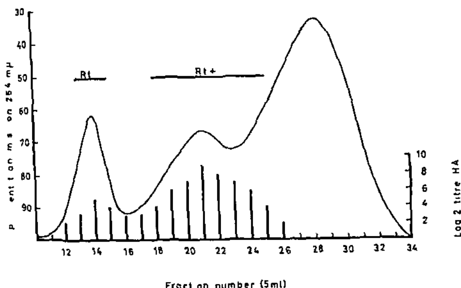


Fig 2
Fractionation by gel filtration on Sephadex G 200 of 2 ml of antiserum to whole bacteria strain F1
HA = Indirect hemagglutination Rt = Ring test.

was 1 8 192 Pre immune serum from the same rabbit gave no precipitation line nor did this serum agglutinate sensitized TFSE

A 2 ml sample of a rabbit immune serum to whole bacteria strain F1 was subjected to gel filtration on Sephadex G 200 The results of the fractionation and of serological tests with the fractions are shown in Fig 2 Precipitating and hemagglutinating antibodies were detected in fractions expected to contain mainly IgM (3) as well as in those containing mainly IgG The highest titres in the hemagglutination tests were obtained with fractions containing mainly IgG The precipitation titres of the fractions were not determined The ring test reaction was very faint with the IgM fractions compared with the reactions seen with fractions containing IgG Treatment with ME resulted in loss of both the precipitating and hemagglutinating capacities of fractions 12 through 15 the titres of the remaining fractions being unaffected by the treatment

Heat Stability of the Antigen

Aliquots containing 0 5 mg/ml of Precipitinogen 2 in phosphate buffered saline were heated at various temperatures for 15 minutes The solutions were then used for sensitization of TFSF for hemagglutination and in hemagglutination inhibition and agar precipitation experiments The results are shown in Table 2 Heating up to 70 C for 15 minutes did not affect the antibody neutralizing capacity or the

nant responsible for precipitation is identical to that which mediates the indirect hemagglutination reaction To 2 ml of a solution containing 1 mg of the antigen in buffered saline was added 0 5 ml of packed TFSE After mixing the suspension was left at 37 C for 30 minutes The supernatant after centrifugation was similarly absorbed two more times The sensitized erythrocytes obtained by each of these absorptions were used in hemagglutination tests with homologous antiserum and the final supernatant was used for agar precipitation and inhibition of hemagglutination One absorption was sufficient to remove detectable amounts of erythrocyte sensitizing antigen In agar precipitation tests the dose of absorbed antigen necessary to give a visible precipitation line was approximately four times that of the unabsorbed control The minimal inhibitory dose in the hemagglutination inhibition test was also four times that of the control (Table 2 bottom row)

Absorption of antiserum with sensitized TFSE was attempted but was found to be impracticable The antiserum could not be diluted more than 1 8 or 1 16 for such experiments and large quantities of antigen therefore were needed for absorption

Two ml of blood was collected from a rabbit s marginal ear vein into 8 ml of Alsever s solution After washing and treatment with tannic acid 1 40 000 the erythrocytes were sensitized with Precipitinogen 2 in the usual manner and finally washed 5 times Immune serum to whole bacteria diluted 1 32 768 agglutinated the sensitized rabbit erythrocytes Approximately 0 1 ml of packed sensitized erythrocytes were suspended in 0 5 ml of saline and injected intravenously into the rabbit A sample of serum collected on the 8th day after the injection gave a line on agar precipitation against a solution containing 0 1 mg/ml of the antigen The line gave a reaction of identity with the line obtained with antiserum to whole microorganisms Serum obtained prior to immunization gave no line in agar with the antigen (Fig 1)

DISCUSSION

Precipitinogen 2 proved to be a complete antigen in all respects It reacted strongly in indirect hemagglutination complement fixation and precipitation tests and induced the formation of antibodies in rabbits

The indirect hemagglutination test using tanned sheep erythrocytes was a very sensitive method for the detection of antibody In experiments currently in progress this reaction is used for the detection of antibodies to Precipitinogen 2 in human sera

The antibody response to injection of the purified antigen has not been studied in detail Rabbit antiserum to whole bacteria contained hemagglutinating and precipitating antibodies of both the IgM and IgG classes This antiserum was obtained 4 weeks after the first injection

Ample time therefore had elapsed for the development of IgG antibody (13) The precipitation reaction was not sufficiently strong to allow for determination of precipitation titres in the unconcentrated fractions obtained by gel filtration Agglutination reactions usually tend to favour the detection of IgM class antibody (13) Nevertheless in the indirect hemagglutination test with Precipitinogen 2 most of the antibody reactivity was found to reside in the IgG fractions In the rabbit protein antigens usually have been found to induce the formation of predominantly IgG class antibody after the early IgM response has decreased (1 4 11 13) Lipopolysaccharide antigens of enterobacteria on the other hand usually have been reported to give rise to a preponderance of IgM antibodies both in rabbit and in man (1 11 12 13)

Digestion with maltase only partially destroyed the precipitating ability and the hemagglutination inhibiting capacity of the antigen It is furthermore well known that periodate oxidation may affect substances other than carbohydrates Nevertheless the results from periodate oxidation and enzymatic digestions strongly suggest that the polysaccharide component is essential for the precipitation and indirect hemagglutination reactions with Precipitinogen 2

The effect of peptic digestion of the antigen indicates that its ability to sensitize sheep erythrocytes depends on the protein component The ability of the material to inhibit indirect hemagglutination was not significantly reduced by digestion with pepsin however nor was the ability to form visible precipitates in agar with undiluted antiserum completely destroyed although much higher concentrations of antigen were necessary for the latter reaction It was previously reported that the precipitating ability in agar double diffusion of Precipitinogen 2 disappeared after digestion of crude extracts with pepsin (7) Only small amounts of the antigen is present in such extracts however and any residual precipitating activity after peptic digestion probably was too weak to be detected by agar precipitation tests

The composition and structure of the antigenic determinant or determinants remain to be further investigated The possibility that a peptide or even a lipid (9) component may be essential for the reactivity can not be excluded The effect of heating at 100 C on the various serological activities of Precipitinogen 2 as well as the effect of 45 per cent phenol on its precipitating ability as previously demonstrated (7) may indicate that a protein or peptide component is important for the serological reactivity of the antigen

Detectable quantities of antigen possessing the ability to sensitize formalinized tanned erythrocytes were readily removed from a solution of Precipitinogen 2 by absorption with TFSF Only small amounts of precipitating and hemagglutination inhibiting antigen were removed however Furthermore sensitized TFSE bound only small amounts of precipitating and agglutinating antibody These results suggest that only a small proportion of the antigenic molecules processes the ability

to attach itself to tanned erythrocytes This would imply a heterogeneity of the material which so far has escaped detection (8)

A rabbit immunized with sensitized erythrocytes produced antibodies which formed the specific line in agar with Precipitinogen 2 This shows that the antigenic determinant responsible for the precipitation reaction is present on the sensitized erythrocytes and strongly suggests that the same antigenic determinant is responsible for the indirect hemagglutination and precipitation reactions

SUMMARY

Serological and antigenic properties of a purified precipitinogen from a strain of oral fusobacteria have been examined

The material fixed complement and gave precipitation in agar and in ring test tubes at low concentrations It could also be adsorbed to tanned sheep erythrocytes The indirect hemagglutination test provided a sensitive method for the detection of antibody

The purified material was antigenic in rabbits Rabbit immune sera obtained after 4 weeks of immunization with whole bacteria contained IgG as well as IgM antibodies both reactive in precipitation and indirect hemagglutination tests

The same antigenic determinant or determinants appears to be responsible for both the precipitating and the hemagglutinating activities of the antigen The polysaccharide component appears to be essential for the serological reactivity of the antigen whereas the ability to attach to tanned erythrocytes depends on a protein component

REFERENCES

1 *Bauer D C Mathies M J & Stavitsky A B* Sequences of synthesis of γ 1 macroglobulin and γ 2 globulin antibodies and secondary responses to proteins salmonella antigens and phage J exp Med *117* 889–907 1963

2 *Daniel T M Weyand J G M & Stavitsky A B* Micromethods for the study of proteins and antibodies IV Factors involved in the preparation and use for a stable preparation of formalinized tannic acid treated protein sensitized erythrocytes for detection of antigen and antibody J Immunol *90* 741–750 1963

3 *Flodin P & Killander J* Fractionation of human serum proteins by gel filtration Biochim biophys Acta (Amst) *63* 403–410 1962

4 *Grey H M* *Studies on the quality of rabbit bovine serum albumin antibody* following immunization Immunology 7 83–90 1964

5 *Herbert W J* Passive haemagglutination In Weir D M (Ed) Handbook of of experimental immunology Blackwell Scientific Publications Oxford and Edinburgh 1967 p 720 744

6 *Kabat F A & Mayer M M* Experimental immunochemistry Thomas Springfield 2 ed 1961

7 *Kristoffersen T* Immunochemical studies of oral fusobacteria I Major precipitinogens Acta path microbiol scand 77 235–240 1969

8 *Kristoffersen T* Immunochemical studies of oral fusobacteria III Purification of a group reactive precipitinogen Acta path microbiol scand 77 447–456 1969

9 *Kristoffersen T* Immunochemical studies of oral fusobacteria IV Some chemical properties of a group reactive precipitinogen Acta path. microbiol cand

Two strains of *Escherichia coli* NCCT 10 08? and NCTC 10 086 were obtained from National Collection of Type Cultures Laboratory strains of *Salmonella typhi* and *Salmonella paratyphi* B were obtained from Dr A Wormnes Department of Microbiology The Gade Institute Bergen

Preparation of Precipitinogen 2

Precipitinogen 2 was prepared from *Fusobacterium* strain F1 as previously de scribed (17)

Rabbit Immune Sera

Antisera to 11 strains of fusobacteria including strains Fev1 and ATCC 10 953 were prepared as before (16) The bacteria had been formalin killed before immu nization For three of the strains antisera to live bacteria were also produced Anti sera to purified Precipitinogen 2 and to rabbit erythrocytes ensitized with the antigen were also prepared as previously described (19) Antisera to live bacteria of two strains each of *L buccalis* and *B melaninogenicus* were kindly supplied by Dr T Hofstad Laboratory of oral microbiology The Gade Institute Bergen The H O antisera to *S typhi* 901 *S paratyphi A* *S typhimurium* and *S newport* were obtained through the courtesy of Dr A Wormnes Bergen

Absorption of Sera with Bacteria

Bacteria were grown in the appropriate media and washed in phosphate buffered saline One ml of serum diluted 1 8 was absorbed at 37 C for 1 hour with approxi mately 0 5 ml of packed washed bacteria

Human Sera and Gamma Globulin Preparations

Umbilical cord sera from 10 individuals and sera from their respective mothers obtained at the time of birth were kindly supplied by Dr O Tønder Department of Microbiology The Gade Institute Bergen An additional 11 cord sera as well as 99 sera from healthy blood donors were obtained at random from the Serological Laboratory Department of Microbiology The Gade Institute Sera from children of various age groups from 3 months to 15 years were obtained through Dr P J Moe Pediatric Hospital University of Bergen School of Medicine and from Drs O Tøn der and T Hofstad of the Gade Institute None of the sera derived from individuals with a history of recent infectious diseases Human gamma globulin in 1? per cent and 16 per cent solutions (A B Kabi Stockholm) were also used

All sera were inactivated at 56 C for 30 minute

Fractionation and Reductive Cleavage of Antibody

Gel filtration on Sephadex G ?00 and reductive cleavage with ? mercaptoethanol (ME) was performed as previously described (19)

Serological Methods

Serological tests were performed largely as described before (19)

For *indirect hemagglutination* a 2 per cent suspension of tanned sheep erythro cytes (TSE) was sensitized by adding an equal volume of a solution containing 0 1 mg per ml of Precipitinogen 2 in phosphate buffered saline With TSE titra tions were made in 0 5 per cent normal rabbit serum Introductory experiments indicated that the reaction with TSE was somewhat more sensitive than with TFSE for the detection of antibodies in human sera In order to make the results as reproducible as possible the same preparation of antigen was used in all these tests As many sera as possible were assayed simultaneously As a check for the reproducibility a rabbit immune serum was always included in the assay Each serum was tested at least twice and the highest titre obtained was recorded as the titre for that serum

For *double diffusion tests* with human sera and human gamma globulin pre parations 0 8 per cent agarose (Nutritional Biochem Corp *Cleveland*) in a vero nal buffer pH 8 6 ionic strength 0 1 was used in addition to the methods already

described (16) Precipitation lines could be enhanced by flushing the agar or agarose plates for 10 minutes in an 0 01% per cent solution of cadmium acetate in the same buffer after the reaction had taken place according to the method of *Crowle* (7)

EXPERIMENTS AND RESULTS

A Distribution of Precipitinogen 2 among Some Oral and Enteric Bacteria

Precipitation All of the rabbit antisera to 11 strains of fusobacteria formed the specific line with solutions of Precipitinogen 2 Antisera to *L buccalis* and *B melaninogenicus* gave no precipitation line in agar nor did they react with the antigen on ring test precipitation Antiserum to purified Precipitinogen 2 and antiserum to rabbit erythrocytes sensitized with the antigen formed the specific line with suspensions of all of 29 strains of fusobacteria tested Pre immune sera gave no precipitation line with Precipitinogen 2

None of the strains of *L buccalis, B melaninogenicus Veillonella* or *Sphaerophorus necrophorus* studied gave the line Crude extracts (16) from these bacteria gave no precipitation on ring test with antiserum to the purified antigen

Indirect hemagglutination All of the 11 antisera to *Fusobacterium* strains agglutinated TFSE sensitized with Precipitinogen 2 Titres varied from 1 128 to 1 16 384 most sera having a titre of 1 1 024 to 1 4 096 Antisera to formalin killed bacteria tended to have lower titres than antisera to live bacteria of the same strain Pre immune sera gave no reactions

Antisera to *L buccalis B melaninogenicus S typhi* 901 *S typhimurium* and *S newport* diluted 1 2 gave no agglutination of sensitized TSE or TFSE One absorption of antiserum to the purified antigen with strains of fusobacteria reduced the titre of this antiserum in indirect hemagglutination tests with TSE from 1 8 192 to <1 16 in most cases With four strains the titre was reduced to ≦ 1 128 by one absorption No reduction of the titre was seen when live *Salmonella E coli Sph necrophorus L buccalis B melaninogenicus* or *Veillonella* were used for absorption

B Antibodies in Human Sera

Indirect hemagglutination The results of indirect hemagglutination tests with different human sera have been compiled in Table 1 Fifteen out of 21 cord sera gave titres of 1 8 or higher and in some instances titres were relatively high Only five sera in the age group 3–11 months were tested and none of them agglutinated sensitized TSE One third of the sera in the age groups 1–3 and 4–6 years gave positive reactions in 1 8 dilutions or higher There was an increase in the relative number of positive reactions with age in the age groups 7–10 years and 13–15 years The titres also tended to be higher in the older age

TABLE 1

Antibody to Precipitinogen 2 in Various Human Sera Distribution of Sera According to Titres in Indirect Hemagglutination Tests

Source of serum	Reciprocal of titres in indirect hemagglutination <8	8 + 16	32 + 64	128 + 256	512 + 1 024	2 048 + 4 096	No of sera with titre >8	Total in age group
Umbilical cord	6	1	7	3	2	2	15	21
Children								
3–11 months	5	0	0	0	0	0	0	5
1– 3 years	5	1	0	1	1	0	3	8
4– 6 years	5	0	1	1	0	0	2	7
7–10 years	2	0	1	2	1	1	5	7
13–15 years	2	0	1	4	4	2	11	13
Blood donors								
18–68 years	13	6	36	24	13	7	86	99

groups Approximately 86 per cent of blood donor sera gave positive reactions and about 20 per cent had titres of 1 512 or higher In no instance was a titre higher than 1 4 096 recorded Human gamma globulin 12 per cent and 16 per cent both gave positive reactions in dilutions 1 1 024 to 1 2 048

The titres of mothers and infants sera paralleled each other well Two out of ten mothers sera gave no hemagglutination in dilution 1 8 In both instances the infants serum also gave no reaction In one instance the titre was the same with the infant's and the mother s sera The titres in the remaining seven mothers sera tested were one or two steps higher than in the infants sera

Absorption of two blood donor sera with 0 1 mg of Precipitinogen 2 per ml of serum removed all hemagglutinating antibody from the sera Absorption of these sera with bacteria of strain F1 had the same effect whereas absorption with two *L buccalis* one *Sph necrophorus* or two *Veillonella* strains did not affect the hemagglutination titres

Four blood donor sera and one cord serum were used in hemagglutination inhibition tests A dilution containing 0 25 μg/ml of Precipitinogen 2 completely inhibited the agglutination of sensitized FSE when 8 agglutinating units of these sera were used

Precipitation The possible presence of precipitating antibodies was studied in 7 human sera and 2 commercial gammaglobulin preparations The hemagglutination titres of the sera examined varied from 1 512 to 1 4 096 Solutions containing 0 05 to 1 0 mg/ml of Precipitinogen 2 in phosphate buffered saline pH 7 2 were used in double diffusion tests in agar and agarose plates With 4 of the human sera no precipitation line was seen even after several days incubation Three sera giving titres ≥ 1 1 024 in hemagglutination tests and both gamma globulin preparations formed a precipitation line with Preci

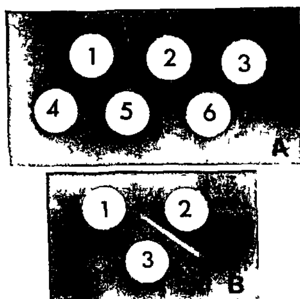


Fig 1

A and B Agar double diffusion plate showing lines formed with Precipitinogen 2 and human gamma globulin Twentyfour hours incubation

A Wells 1 and 2 Human gamma globulin 16 per cent (Kabi)
Well 3 Same but absorbed with Precipitinogen 2
Well 4 PBS
Wells 5 and 6 Precipitinogen 2 0 1 mg/ml in PBS
B Well 1 Human gamma globulin 16 per cent (Kabi)
Well 2 Antiserum to the purified antigen
Well 3 Precipitinogen 2 0 1 mg/ml in PBS

pitinogen 2 in agar and agarose plates (Fig 1 A) The line was rather faint but was clearly visible after 1 to 3 days incubation especially when the plates were flushed for 10 minutes with a buffer containing 0 0125 per cent cadmium acetate With the gamma globulin preparations such enhancement was unnecessary The line showed deviation and fusion with the line formed with the antigen and rabbit immune serum With potent undiluted rabbit antiserum there was a strong spur formation (Fig 1 B) No precipitation line was seen when the sera or gamma globulin preparations had been absorbed with surplus amounts of Precipitinogen 2 nor did unabsorbed sera or gamma globulin preparations give the precipitation line with phosphate buffered saline (Fig 1 A)

The nature of antibodies in human sera Three blood donor sera with high titres in the indirect hemagglutination test as well as a pool of ten such sera were fractionated by gel filtration Fractions were examined in indirect hemagglutination tests Results of a representative gel filtration experiment in which 1 5 ml of a blood donor serum

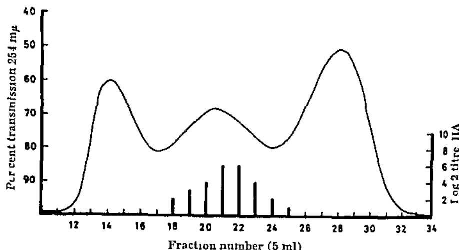


Fig 2

Fractionation by gel filtration on Sephadex G 200 of 1 5 ml serum from a healthy blood donor HA = indirect hemagglutination

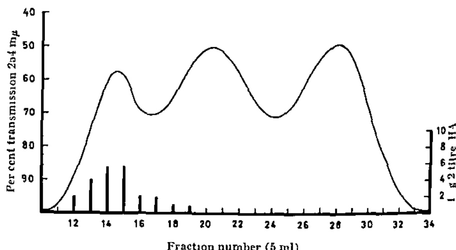


Fig 3

Fractionation by gel filtration on Sephadex G 200 of 1 5 ml of serum from a healthy 15 year old boy HA = indirect hemagglutination

was fractionated are given in Fig 2 The hemagglutinating activity was found in fractions corresponding to the second peak of U V absorbing material eluted from the column The reactivity was not significantly affected by treatment with ME The findings indicate that the anti bodies responsible for indirect hemagglutination with Precipitinogen 2 were primarily of the IgG class

Ten additional sera from individuals from different age groups in cluding two cord sera were subjected to reductive cleavage with ME Only in one instance was the titre reduced by more than one step after

treatment with ME This serum derived from a 15 year old boy Fig 3 illustrates the results of fractionation by gel filtration of this serum The hemagglutinating activity was found to reside in the first peak eluted from the column Fractions 14 and 15 were pooled and concentrated to approximately 2 5 ml by ultrafiltration After treatment with ME of the concentrate no agglutination of sensitized TSE occurred

DISCUSSION

Although only a limited number of strains have been examined it can be concluded that Precipitinogen 2 is widely distributed among oral fusobacteria None of the other bacterial strains examined appeared to contain Precipitinogen 2 nor did antisera to microorganisms other than fusobacteria react with the antigen The number of microbial species and strains tested is not great The results strongly indicate however that Precipitinogen 2 is characteristic of fusobacteria and that it rarely—if ever—occurs in other oral or enteric bacteria This also holds true for the presumably (4 20 25) related *L buccalis* and *Sphaerophorus necrophorus*

By use of the indirect hemagglutination technique antibodies to Precipitinogen 2 could be demonstrated in approximately 86 per cent of human sera from adults and 76 per cent of the cord sera The results of gel filtration experiments and treatment of sera with ME indicate that the hemagglutinating antibodies are primarily of the IgG class The frequent occurrence of antibodies in cord sera is compatible with this finding Furthermore there was very little difference between hemagglutination titres in mothers and infants sera at birth

Hemagglutinating antibodies were detected as early as in one year old children At this age and even from the fifth month of life fusobacteria were found by *Berger et al* (1) to be regularly present in children s mouths

The number of children s sera examined is small and several age groups were not represented There appeared however to be an increase in the incidence of positive reactions as well as a tendency for titres to be higher with age The distribution of titres in various age groups over 18 years were not further investigated The studies of *Evans et al* (9) indicated that an investigation of the relationship between titre levels and the incidence and severity of marginal periodontal disease would be more relevant *Evans et al* (9) found significantly elevated titres of bactericidal antibody to fusobacteria in sera from 6 individuals with periodontal disease as compared with 5 normal sera Nothing was known about the condition of the periodontal tissues in our material Investigations on the titres of serum antibody to Precipitinogen 2 in individuals in various age groups for whom the health status of the periodontal tissues is known are in progress

Precipitation reactions between Precipitinogen 2 and human sera and gamma globulin preparations were weak A systematic search for sera with higher levels of precipitating antibodies has not been carried out The spur formation between the precipitation lines with rabbit immune and human sera (Fig 1 B) may reflect differences in sero logical specificities or merely unbalanced systems (8 23)

Is is not known how Precipitinogen 2 has gained access for antigenic stimulation A great number of investigations have pointed out that the number of Gram negative bacteria among them fusobacteria is both relatively and absolutely increased in inflammatory periodontal disease (15 22 27 28 29) In this situation the bacteria are in close contact with the ulcerated or degenerated epithelium overlying the in flamed tissues (31) This would appear to provide ready access for bacterial antigens to the highly vascular gingival connective tissue The findings of *Evans et al* (9) support this view That bacterial pro ducts can penetrate into the tissues by this route in rabbits was re cently shown by *Rizzo* (24) Other reports indicate that this may also occur in humans (6 9 26 30) The dense accumulation of plasma cells containing IgG and IgA and the high concentration of these immuno globulins in the connective tissue ground substance (2) has been inter preted as a local release of specific antibody against components of the gingival bacterial plaque (3) Gingival inflammation and destruc tion of periodontal tissues may occur very early in life and is fre quently seen in the temporary dentition (14) The epithelium of the gingival crevice area also has been considered by some to be parti cularly vulnerable during and for some time after the eruption of teeth (5)

The possibility can not be excluded however that the antigen may have gained access to the interior of the body through other parts of the gastro intestinal or the nasopharyngeal tracts Fusobacteria have been reported to be found in many locations of the body outside the oral cavity (for reviews see (4 25)) *Lahelle* (21) reported the pre sence of complement fixing antibody to fusobacteria in a patient with a necrotic fusospirochetal infection in a finger *Bøe* (4) also found antibodies in humans with abscesses caused by fusobacteria

A detailed discussion of the possible clinical significance of circu lating antibodies to oral fusobacteria e g in relation to periodontal disease is beyond the scope of this paper Perhaps the most inter esting aspect is the possible influence of the reactions of these anti bodies and corresponding antigens on local tissue damage in bacterial inflammation Such damage could conceivably be brought about by several mechanisms as indicated for instance by the recent findings of *Gustafson et al* (10 11) and *Gustafson* (12) In animal experiments using Protein A from *Staphylococcus aureus*, they found evidence that circulating natural and aquired precipitating antibodies may play a part not only in anaphylaxis like and Arthus reactions but in Schwartz

man like haemorrhagic reactions as well (for review see *Gustafson* (13)) Whether or not Precipitinogen 2 and corresponding antibodies may be capable of evoking similar reactions merits further investigations Such reactions might present interesting experimental models in the study of immune injury particularly with regard to inflammatory periodontal disease

SUMMARY AND CONCLUSIONS

All of 29 strains of fusobacteria reacted with specific antiserum to Precipitinogen 2 in precipitation and indirect hemagglutination tests Similarly antisera to all of 11 of these strains reacted with the purified antigen None of ten other species of oral and enteric bacteria or corresponding antisera gave such reactions It was concluded that Precipitinogen 2 is a group specific antigen which is widely distributed among oral fusobacteria

By an indirect hemagglutination technique antibodies to the antigen were found in 86 per cent of sera from adult humans tested as well as in 76 per cent of human cord sera Antibodies were found in sera of children from one year of age The incidence of positive reactions and the titre levels appeared to increase with age in children and adolescents

Precipitating antibodies to Precipitinogen 2 were found in some of the human sera as well as in commercial preparations of human gamma globulin

REFERENCES

1 *Berger U Kapovits M & Pfeifer G* Zur Besiedlung der kindlichen Mundhohle mit anaeroben Mikroorganismen Z Hyg Infekt kr *145* 564–573 1959
2 *Brantzæg P & Kraus F W* Autoimmunity and periodontal disease Odont T 73 281–393 1965
3 *Brantzæg P* Local factors of resistance in the gingival area J periodont Res *1* 19–42 1966
4 *B e J* *Fusobacterium* Studies on its bacteriology serology and pathogenicity Skr Norske Vitensk Akad I Mat nat Kl no 9 Oslo 1941
5 *Cohen B* Morphological factors in the pathogenesis of periodontal disease Brit dent J *107* 31–39 1959
6 *Courant P R & Bader H* *Bacteroides melaninogenicus* and its products in the gingiva of man Periodontics 4 131–136 1966
7 *Crowle A J* Enhancement by various cations of the double diffusion precipitation test Int Arch Allergy *16* 113–125 1960
8 *Crowle A J* Immunodiffusion Academic Press New York and London 19 1
9 *Evans R T Spaeth S & Mergenhagen S E* Bactericidal antibody in mammalian serum to obligatorily anaerobic gram negative bacteria J Immunol *97* 112–119 1966
10 *Gustafson G T Sjoquist J & Stålenheim G* Protein A from *Staphylococcus aureus* II Arthus like reaction produced in rabbits by interaction of Protein A and human γ globulin J Immunol *98* 1178–1181 1967
11 *Gustafson G T Stalenheim G Forsgren A & Sjoquist J* Protein A from *Staphylococcus aureus* IV Production of anaphylaxis like cutaneous and systemic reactions in non immunized guinea pigs J Immunol *100* 530–534 1968

12 *Gustafson G T* Hemorrhagic reactions in the hamster produced by interaction of Protein A from *Staphylococcus aureus* human γ globulin and endotoxin Acta path microbiol scand 7, 127–138 1968
13 *Gustafson G T* Hypersensitivity mechanisms in bacterial inflammation with special reference to periodontal disease Acta path. microbiol scand 74 Suppl 196 1968
14 *Jamison H C* Prevalence and severity of periodontal disease in a sample of a population Thesis Ann Arbor University of Michigan School of Public Health 1960
15 *Jensen S B* En undersogelse af gingivas bakterieflora ved forskellige former for marginale paradentopatier Tandlægebladet 62 9–30 1958
16 *Kristoffersen T* Immunochemical studies of oral fusobacteria I Major precipitinogens Acta path microbiol scand 77 235–246 1969
17 *Kristoffersen T* Immunochemical studies of oral fusobacteria III Purification of a group reactive precipitinogen Acta path microbiol scand 77 447–456 1969
18 *Kristoffersen T* Immunochemical studies of oral fusobacteria IV Some chemical properties of a group reactive precipitinogen Acta path microbiol scand 77 457–464 1969
19 *Kristoffersen T* Immunochemical studies of oral fusobacteria V Serological characterization of a group reactive antigen Acta path microbiol scand 77 707–716 1969
20 *Lahelle O* *Necrobacterium* A study of its bacteriology serology and pathogenicity and its relation to *Fusobacterium* Acta path microbiol scand Suppl 67 1947
21 *Lahelle O* Finger infection caused by *Fusobacterium* and spirochetes with a discussion of fusospirochetal infection of the fingers Acta derm venereol 25 266–274 1945
22 *Löe H Theilade E Jensen S B & Schiott C R* Experimental gingivitis in man J Periodont 36 177–187 1965
23 *Ouchterlony Ö* Diffusion in gel methods for immunological analysis Progress in Allergy 6 30–154 1962
24 *Rizzo A A* Absorption of bacterial endotoxin into rabbit gingival pocket tissue Periodontics 6 65–70 1968
25 *Rosebury T* Microorganisms indigenous to man McGraw Hill Book Co Ltd. New York 1962
26 *Schneider T F Toto P D Gargiulo A W & Pollock R J* Specific bacterial antibodies in the inflamed human gingiva Periodontics 4 5 –57 1966
27 *Schultz Haudt S D Bruce M A & Bibby B G* Bacterial factors in nonspecific gingivitis J dent Res 33 454–458 1954
28 *Socransky S S Gibbons R J Dale A C Bortnick L Rosenthal E & Macdonald J B* The microbiota of the gingival crevice area of man I Total microscopic and viable counts and counts of specific organisms Arch oral Biol 8 275–280 1963
29 *Theilade E Wright W H Jensen S B & Löe H* Experimental gingivitis in man II A longitudinal clinical and bacteriological investigation J periodont Res 1 1–13 1966
30 *Tsutsui M Usumi N & Tsubakimoto K* Cellular components of staphylococci and streptococci in inflamed human gingiva J dent Res 47 663 1968
31 *Wærhaug J* The gingival pocket Odont T 60 Suppl 1 1952

Acta path microbiol. scand 77 727–738 1969

The Institute of Medical Microbiology Department of Bacteriology and the Department of Pediatrics University of Goteborg Goteborg Sweden

IMMUNODIFFUSION STUDIES ON *ESCHERICHIA COLI*

2 *Characterization of Antigens Used for Passive Haemagglutination with an O 6 Strain as Model*

By

JAN HOLMGREN and LARS Å HANSON

Received 17 vi 69

In many earlier investigations of the antibody response to *E coli* in patients with enteric (*i a* 14 15) and urinary tract infections (*i a* 1 2 4 6 12 18 21 25) passive haemagglutination techniques have been used These studies have employed preparations from *E coli* strains which have not been analyzed in detail for their antigenic composition

In a previous work using immunodiffusion techniques a highly purified O antigen prepared according to the method of *Westphal et al* was found to be contaminated with K antigen (7) It therefore seemed to be of interest to characterize further different types of antigen preparations used as O antigen in passive haemagglutination titrations as well as to investigate the erythrocyte sensitizing capacity of K antigen Such a characterization is necessary for the interpretation of results obtained by passive haemagglutination titrations of antibodies to *E coli* O antigen The present paper is part of a study of the immune response in children with urinary tract infections caused by *E coli* (1 2 3 6 7 25)

MATERIALS

Bacterial Strains

The following *E coli* O antigen test strains kindly supplied by Drs I and F Ørskov at the WHO International Escherichia Centre Statens Seruminstitut Copenhagen were used

Strain designation	Serotype		
	O	K	H
U5 41	1	1	7
U9 41	2	1	4
U4/41	4	3	5

Thanks are due to Miss *Birgitta Berglund* and Miss *Ingela Svärdström* for skilful technical assistance

This work was supported by grants from the *Swedish Medical Research Council* (K 67—19 X—215—04 A) and the *Faculty of Medicine Göteborg*

Strain designation	Serotype		
	O	K	H
Bi 7458/41 (the model strain)	6	2a 2c	1
Su 4344/41	6	13	1
Bi 7509/41	7	1	–
G 3404/41	8	8	4
Su 4411/41	14	7	–
F 10018/41	18	76	14
E 3b	75	?	5

All of these strains were of the smooth colony type The O 6 K 2a 2c H 1 strain was used as the *model strain* for the detailed analyses since its antigens had previously been extensively analyzed (7)

Antigen Preparations

Stock suspensions of bacteria were obtained as earlier described (7) at densities corresponding to 50 mg of acetone dried bacteria/ml

Heated antigen (HA) The bacterial stock suspensions of the two O 6 strains and the O 14 strain were heated to 100 C for 2 hours After centrifugation (3000 rpm 30 min) the supernates were used as antigens (HA)

Common antigen An aliquot of HA from *E coli* O 14 was fractionated into O antigen and the common antigen of *Kunin Beard & Halmagyi* (9) by precipitation with 85 per cent ethanol according to the method of *Suzuki Gorzynski & Neter* (19)

Purified lipopolysaccharide (LPS antigen) prepared according to *Westphal et al* (24) from *E coli* O 1 O 2 O 4 O 6 (the model strain) O 7 O 8 O 14 O 18 and O 75 were kindly supplied by Drs B and K Jann Max Planck Institut fur Immunbiologie Freiburg These protein free preparations were designated *O 1 LPS O 2 LPS* etc They were used at a concentration of 1 mg/ml From previous double diffusion analyses (7) the O 6 LPS was known to give three precipitates with O (see also Fig 4) and four with OKH antiserum to the model strain the fourth precipitate representing contaminating K 2a 2c antigen

Veronal buffer extract antigen (VE antigen) was prepared from the model strain O 6 K 2a 2c H 1 as described previously (7) and used at a concentration of 30 mg/ml This preparation was characterized in some detail in a previous study (7)

The alkali treated and the heat treated preparations of VE and LPS antigens from the model strain The alkali treatment was performed according to Jann (8) To 0 5 ml of VE antigen (or LPS at 1 mg/ml) 1 ml of 0 25 N NaOH was added and the mixture was incubated at 56 C for 30 minute whereupon 0 25 N HCl was added until neutral pH was attained The solution was centrifuged (3000 rpm 30 min) and the supernate concentrated to the original volume *The heat treatment* consisted of boiling for 2 hours

Purified K antigen The K antigen (K 2a 2c) from the model strain was obtained purified by preparative zone electrophoresis in Sephadex G 25 (7) It was not contaminated by other antigenic material according to immunodiffusion analysis (7) and was used at a concentration of 1 mg/ml Heated K 2a 2c was prepared by boiling a portion of purified K 2a 2c antigen for 2 hours

Antisera

OKH and *O antisera* against *E coli* O 6 K 2a 2c H 1 (the model strain) and against O 6 K 13 H 1 and O 14 K 7 H were produced in rabbits as described earlier (7) Only O antisera were prepared against the other aforementioned strains

METHODS

Double diffusion electrophoretic immuno electrophoretic and comparative immuno electrophoretic analyses were performed as reported (22 23) The double diffusion immuno electrophoretic and electrophoretic plates were stained with amidoblack or azocarmin

Passive haemagglutination titrations were performed according to *Neter et al* (13) as earlier described (25)

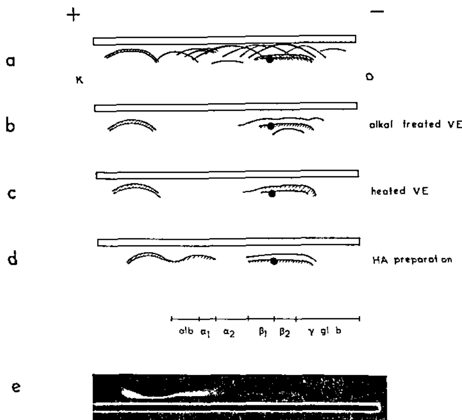


Fig 1

Immuno electrophoretic patterns developed by untreated alkali treated and heated VE antigen (a–c) and HA preparations (d) from the model stra n w th OKH anti serum against this strain (longitudinal basins) The electrophoretic distribution of human serum proteins is in luded for reference purposes The photo (e) shows an analysis similar to that visualized n d

RESULTS

Characteri ation of Antigenic Preparations from the Model Strain

Comparison of all ali and heat treated VE antigen with the un treated VE antigen Untreated alkali treated and heated VE antigens from the model E coli strain (O 6 K 2a 2c H 1) were compared by immuno electrophoresis (Figs 1a–c) With anti O 6 K 2a 2c H 1 OKH immune ser a the alkali treated VE antigen gave an immuno electro phoretic pattern much less complex th in the untreated VE antigen In both patterns a broad precipitate formed by O antigen (7) and consistin of at least two parallel lines was observed in the region corresponding to that of human serum β globulins (Figs 1a–b) One of the lines formed by O antigen in the alkali treated VE antigen had a second curvature nearer the cathode In addition paralle'

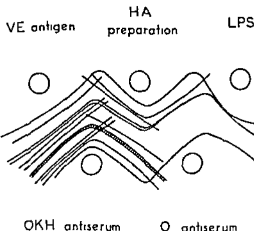


Fig 4

Double diffusion patterns of HA preparations from the model strain with OKH and O immune sera against this strain compared with patterns formed by VE antigen and LPS from the same strain with the OKH and O immune sera respectively

TABLE 1

The Erythrocyte Sensiti ing Capacity of O Antigen Containing Preparations from the Model Strain

Model strain O 6 K 2a 2c H 1	Passive haemagglutination Titre (reciprocal) with O antiserum No 1	No 2	No 3	Protein stained material in the O antigen region at agar gel electro phoresis (cf Fig 2)
VE antigen 1/10 000				
Untreated	< 8	< 8	< 8	+
Alkali treated	8 192	32 768	32 768	—
Heat treated	2 048	8 192	4 096	—
O 6 LPS 1/100 000				
Untrated	< 4	< 4		
Alkali treated	< 4	< 4		
Heat treated	< 4	< 4		
O 6 LPS 1/10 000				
Untreated	256	2 048		
Alkali treated	4 096	32 768		
Heat treated	1 024	8 192		
O 6 LPS 1/1 000				
Untreated	2 048	32 768		
Alkali treated	4 096	131 072		
Heat treated	8 192	32 768		
O 6 LPS (Undiluted)				
Untreated	2 048	65 536		—
Alkali treated	8 192	32 768		—
Heat treated	8 192	65 536		—

All electrophoretic separations were made with undiluted materials

Analysis of this preparation with OKH antisera gave a pattern rather similar to that obtained with the heated VE antigen from the same strain (Figs 1c and d) However the precipitate demonstrated to be formed by K antigen in the HA was slightly more cathodically lo

cated than the corresponding precipitate obtained with the heated VE antigen and it also had a second maximum closer to the start basin (Fig 1d)

Comparative double diffusion analysis of the HA VF and LPS preparations was also performed (Fig 4) HA gave at least eight lines with OKH antisera against the model strain i e a pattern more multi linear than that observed in immuno electrophoresis With O antisera against this strain however the number of lines developed by HA was only four (Fig 4) Many of the antigenic factors in HA were identical with factors in the VF antigen All of the four antigenic factors in the LPS were also found in HA One of these formed a line with OKH but not with O antisera and was identified as K antigen

The Erythrocyte Sensitizing Capacity of Antigenic Preparations from the Model Strain

Comparison of alkali and heat treated VE antigen with the un treated VE antigen By tenfold serial dilutions untreated VE antigen from the model strain (O 6 K 2a 2c H 1) was diluted to the first di lution (1/10 000) that could no longer sensitize sheep erythrocytes to give visible agglutination with rabbit O antisera to *E coli* O 6 K 2a 2c H 1 Erythrocytes of the same batch coated with the same dilutions of alkali treated or heat treated VF antigen from the same strain were agglutinated by the same antisera at high titres see Table 1 The titre obtained with a certain serum using erythrocytes coated with the alkali treated VE antigen was consistently four to eightfold higher than that obtained with erythrocytes coated with the heated VE antigen

Comparison of alkali and heat treated LPS with the untreated LPS By tenfold serial dilutions untreated O 6 LPS (1 mg/ml) was diluted to the first dilution that could no longer sensitize sheep red blood cells to give visible agglutination with rabbit O antisera to the model strain Red blood cells of the same batch were then incubated with the same dilutions of alkali treated or heated LPS and after washing the incubated cells were used in passive haemagglutination titrations of the O antisera Some differences in the erythrocyte coating capacity of these three preparations were observed although they were not of the magnitude observed in the similar experiment with the VE anti gen (Table 1)

The K 2a 2c antigen used for passive haemagglutination The ca pacity of isolated K 2a 2c antigen to coat erythrocytes for passive haemagglutination was investigated High dilutions (1/1 000–1/80 000) of the rabbit OKH antisera to *E coli* O 6 K 2a 2c H 1 agglutinated sheep red blood cells coated with the isolated K 2a 2c antigen while even undiluted OKH antisera to O 6 K 13 H 1 with high anti O 6 antibody titres gave no such agglutination The K antigen specificity

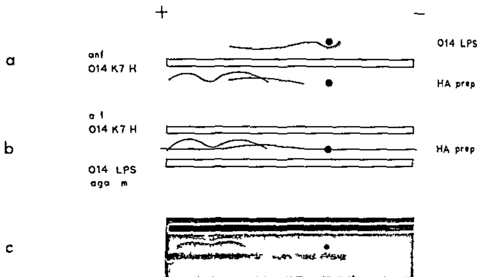


Fig 5

Immuno electrophoretic (a) and comparative immuno electrophoretic (b) patterns formed by O 14 LPS and HA preparation from O 14 K 7 H with OKH antiserum against this strain The photo (c) shows an analysis similar to that depicted in b

of these agglutinating antibodies was further tested by absorbing anti O 6 K 2a 2c H 1 OKH immune sera with erythrocytes coated with HA from *E coli* O 6 K 13 H 1 These absorbed antisera did not contain agglutinins to the O 6 antigen as shown by control experiments but they still agglutinated erythrocytes coated with isolated K 2a 2c antigen at the same titres as before the absorption After boiling for two hours the K 2a 2c antigen still had practically full effect in sensitizing red blood cells for passive haemagglutination

Characterization of Antigenic Preparations of E coli *O 14*

E coli O 14 LPS was investigated with immunodiffusion techniques It gave two precipitation lines with OKH as well as O antisera against *E coli* O 14 K 7 H in double diffusion analyses In immuno electrophoresis the *E coli* O 14 LPS with OKH and O antisera formed a precipitate with a maximum in the region corresponding to that of human serum α_1–α globulin region with the cathodical end curving around the start basin (Fig 5a)

HA from *E coli* O 14 K 7 H gave three precipitates with O and OKH antisera against this strain in double diffusion analyses Two of these precipitating factors were also present in the O 14 LPS while the third was not In regular (Fig 5a) and comparative (Fig 5b) immuno electrophoresis HA from the O 14 strain formed two precipitates with homologous O and OKH antisera One of these precipitates was localized in the region corresponding to that of human serum α_1–α globulins and showed antigenic identity with O 14 LPS in com

parative immuno electrophoresis The other precipitate extended into the pre albumin region had two maxima and was *unaffected by the* LPS antigen in the comparative analysis (Fig 5b) Absorption of O 14 antiserum with O 14 HA completely removed the precipitating antibodies to O 14 LPS as shown by immuno electrophoresis

Fractionation of O 14 *HA* with 85 per cent ethanol was performed to separate the O antigen and the common antigen of *Kunin et al* The ethanol insoluble fraction gave the same immuno electrophoretic pattern with O 14 antisera as the HA while the ethanol soluble fraction (common antigen) was nonprecipitinogenic O antisera against *E coli* O 14 did not precipitate with LPS from coli strains of any of the O groups 1 2 4 6 7 8 18 and 75 O antisera against these eight O groups did not precipitate with HA or LPS from the O 14 strain employed

DISCUSSION

In studies of the antibody response in patients with *E coli* infections of the urinary tract the passive haemagglutination techniques is often employed (1 2 4 6 12 18 21 25) In most studies O antigens for coating of the red cells have been prepared by boiling suspensions of the infecting strain Such crude O antigen preparations have not been analyzed in detail for antigenic composition In the present study a *veronal buffer extract* (VE) antigen from an *O 6 strain* containing at least 14 antigenic factors which had been ascertained by immuno diffusion methods (7) was used for comparison with a boiled suspension of the same O 6 strain This heated preparation (HA) contained at least eight antigenic factors Three of these identified with those three factors in the purified O 6 lipopolysaccharide which were precipitated by O 6 O antisera Another factor in the boiled O 6 antigen was identified as K antigen The possible occurrence of partly heatstable K antigens in addition to O antigens in HA preparations *has been earlier suggested* (3) The nature of the remaining four factors is unknown The observation that some of the factors in the HA did not identify with factors in the VE antigen (Fig 4) might be due to denaturation of antigens during heating The heating may also extract or uncover antigens which are not obtained by the mild action of the veronal buffer In immuno electrophoresis the HA preparation gave two main precipitates one of which was formed by O antigen and the other by K antigen (Fig 1d) The form and localization of the O 6 antigen precipitate was similar to that obtained with purified O *antigens or heat extracts from bacteria of the eight E coli O groups* most prevalent in urinary tract infections (7) The four factors in the HA which were unrelated to the O and K antigens were not seen in immuno electrophoresis although they were visible in double diffusion analyses This is probably due to their presence in amounts

too small to be revealed by the less sensitive immuno electrophoretic method

The HA preparations which are commonly used in passive haemagglutination tests obviously contain O antigen as a main factor K antigens however may also be present An isolated K antigen was found to coat sheep red blood cells so that they were agglutinated by anti K antibodies A similar observation has been reported by *Ørskov et al* (16 17) The antibodies demonstrated in earlier studies of patients with urinary tract infection using boiled bacterial suspensions as antigen have been regarded mainly as anti O antibodies (*i a* 1 3 4 21) In view of the present findings and in accord with earlier suggestions (3 17) some of these antibodies may be anti K antibodies It has been observed in some cases that patients with a recurrent urinary tract infection show a titre rise against the *E coli* strain which was isolated during the preceding infection although the new infection was caused by an *E coli* strain of another O group (1) In some cases this could be due to the presence of the same or cross reacting K antigen in the two strains The second antibody rise demonstrated with HA preparations could then in fact have been due to anti K antibodies Further studies are needed to elucidate this aspect

An O group of special interest as regards the specificity of passive haemagglutination titration is O 14 since Kunin and his associates (9 10) have shown that bacteria of this group are rich in an antigen common to most *E coli* O groups Immuno electrophoretic analyses of an HA preparation of the O 14 strain revealed one fast moving factor possibly corresponding to K antigen and a second factor identified as O 14 antigen (cf Fig 5b) This O 14 antigen had a higher electrophoretic mobility however than eight other different O antigens previously studied (7) This mobility difference might be due to the comparatively large amounts of the common antigen being associated with the O 14 antigen as suggested by *Kunin et al* (9) However ethanol fractionation to remove the common antigen (19) did not change the immuno electrophoretic pattern of O 14 HA with O 14 antisera We have not obtained precipitates with the O 14 antigen and O antisera to any of the O groups 1 2 4 6 7 8 18 and 75 or between anti O 14 and O antigens from test strains of these eight O groups This lack of cross reaction supports the observation by *Kunin et al* (9) that the common antigen is not precipitinogenic By haemagglutination inhibition experiments *Andersen* has shown that the common antigen does not significantly influence the passive haemagglutination titres obtained against boiled suspensions of the infecting *E coli* strains in children with urinary tract infection (2)

It is well known that lipopolysaccharide but not protein antigens easily attach to the surface of untreated red blood cells (5) Heat or alkali treatment of O antigen preparations has been used to improve

the capacity of the antigen to sensitize red blood cells for passive haemagglutination (5 11) Parallel to the improvement of the sensitizing capacity of our veronal buffer extract of *E coli* by the heat or alkali treatment a loss of protein staining material was observed Furthermore a protein free purified lipopolysaccharide did not sensitize red cells much more efficiently after heat or alkali treatment Possibly the improved attachment of the O antigens to the red cells was as has also been suggested for *Salmonella* O antigens (20) partly due to the elimination of protein hindering the attachment The less improvement obtained by heat or alkali treatment of the purified lipopolysaccharide may be due to additional changes as suggested by *Neter et al* (15)

SUMMARY

Antigens for passive haemagglutination assays prepared from an O 6 strain were characterized by immunodiffusion methods The most commonly used O antigen containing preparation the supernate of a boiled bacterial suspension comprised at least eight antigenic factors three of which were related to O antigen This preparation as well as the purified O 6 lipopolysaccharide contained some K antigen This K antigen sensitized sheep red blood cells for passive haemagglutination In the light of this finding the possible relevance of using such O antigen preparations in the study of the antibody response to *E coli* is discussed

Studies of an O 14 strain did not reveal the common antigen of *Kunin et al* as a precipitinogenic factor

Heat or alkali treatment used to increase the red cell sensitizing capacity of *E coli* antigen preparations simultaneously decreased the protein content of the antigens

REFERENCES

1 *Andersen H J* Clinical studies on the antibody response to *E coli* O antigens in infants and children with urinary tract infection using a passive hemagglutination technique Thesis Acta Paed Scand Suppl *180* 1968

2 *Andersen H J* Studies of urinary tract infections in infancy and childhood VII The relation of *E coli* antibodies in pyelonephritis as measured by homologous and common (Kunin) antigens J Paediat *68* 542–550 1966

3 *Andersen H J Hanson L A Lincoln K Orskov F & Winberg J* Studies of urinary tract infections in infancy and childhood IV Relation of the coli antibody titre to clinical picture and to serological type of the infecting *Escherichia coli* in acute *uncomplicated urinary tract infections* Acta paediat scand 54 247–259 1965

4 *Ehrenkranz N J & Carter M J* Immunologic studies in urinary tract infections I The hemagglutinin response to *Escherichia* O antigens in infections of varying severity J Immunol 92 798–806 1964

5 *Goodman H C & Bozicevich J* Recently developed techniques for the use of inert particles to detect antigen antibody reactions Immunological methods (Ed J F Ackroyd) pp 93–109 Blackwell Scientific Publications Oxford 1964

6 *Hanson L A & Winberg J* Demonstration of antibodies of different immunoglobulin types of the O antigen of the infecting *E coli* strain in infants and children with pyelonephritis Nature *212* 1495–1496 1966
7 *Holmgren J Eggertsen G Hanson L A & Lincoln K* Immunodiffusion studies on *Escherichia coli* 1 Identification of O K and H antigens in an O 6 strain *Acta path microbiol scand 76 304–318 1969*
8 *Jann K* Personal communication 1967
9 *Kunin C M Beard M V & Halmagyi N* Evidence for a common hapten associated with endotoxin fractions of *E coli* and other *Enterobacteriaceae* Proc Soc exp Biol N Y *111* 160–166 1962
10 *Kunin C M* Separation characterization and biological significance of common antigen of *Enterobacteriaceae* J Immunol 74 466–478 1955
11 *Landy M* On hemagglutination procedures utilizing isolated polysaccharide and protein antigens Amer J publ Hlth *44* 1059–1064 1954
12 *Needell M H Neter E Staubit W J & Bingham W A* The antibody (hemagglutinin) response of patients with infections of the urinary tract J Urol *74* 674–682 1955
13 *Neter E Bertram L F Zal D A Murdock M R & Arbesman C E* Studies on hemagglutination and hemolysis by *Escherichia coli* antisera J Exp Med. *96* 1–12 1952
14 *Neter E & Suskind L* The antibody (hemagglutinin) response as a tool for etiologic diagnosis of diarrheal disease J Pediat *52* 38–41 1958
15 *Neter E Westphal O Lüderit O Gorzynski E A & Eichenberger E* Studies of enterobacterial lipopolysaccharides Effects of heat and chemicals on erythrocyte modifying antigenic toxic and pyrogenic properties *J Immunol 76* 377–385 1956
16 *Orskov I & Orskov F* What is the nature of *E coli* K antigens? An attempt to analyse the K antigens by various serological methods Arch Immunol Ther Exptl *16* 387–391 1968
17 *Orskov I Orskov F Jann B & Jann K* Acidic polysaccharide antigens of a new type from *E coli* capsules Nature *200* 144–146 1963
18 *Slifkin M Ward L M Fischer J J & Cromartie W J* Value of serological *studies in the diagnosis of chronic infections of the* urinary tract Bact Proc *98* 1959
19 *Suzuki T Gorzynski E A & Neter E* Separation by ethanol of common and somatic antigens of *Enterobacteriaceae* J Bact *88* 1240–1243 1964
20 *Truszczynski M* Immunological characterization of antigenic extracts obtained from bacteria with different methods I Microorganisms of the genera *Salmonella* and *Escherichia* Arch Immunol Ther Exptl *11* 241–271 *1963*
21 *Vosti K L Monto A S & Rant L A* Host parasite interaction in patients with infections due to *Escherichia coli* II Serologic response of the host J Lab Clin Med *66* 613–626 1965
22 *Wadsworth C* A slide microtechnique for the analysis of immune precipitates in gel Int Arch Allergy *10 355–360* 1957
23 *Wadsworth C & Hanson L A* Comparative analysis of immune electrophoretic precipitates employing a modified immune electrophoretic technique Int Arch Allergy *17* 165–177 1960
24 *Westphal O Luderit O & Bister F* Ueber die Extraktion von Bakterien mit Phenol/Wasser Z Naturforsch *76* 148–155 1952
25 *Winberg J Andersen H J Hanson L A & Lincoln K* Studies on urinary *tract* infections in infancy and childhood I Antibody response in different types of urinary tract infections caused by coliform bacteria *Brit Med J II* 524–527 1963

Acta path microbiol scand 77 739–749 1969

The Department of Experimental Ophthalmology (Head Prof C E T Krakau)
and Department of Medical Microbiology
(Head Prof R Grubb) University of Lund Lund Sweden

QUANTITATION OF ENDOTOXIN INDUCED PROTEIN INCREASE IN THE AQUEOUS HUMOR OF THE RABBIT'S EYE

By

H Kjell Dyster Aas Hans Fritz and C E Torsten Krakau

Received 7 vi 69

In rabbits endotoxinaemia is followed by a condition resembling the clinical entity of acute iritis with an increase of the protein concentration of the aqueous humor hyperaemia and a transitory increase in intraocular pressure (*Ayo & Meyer* 1942 *Ayo* 1943 *Levene & Breinin* 1959 *Skrzypczal* 1968)

The aqueous flare is a Tyndall phenomenon the density of which reflects the protein content of the aqueous humor The normal protein concentration of the aqueous humor is very low in rabbits about 0 5–1 0 per cent of that in the blood Impairment of the blood aqueous barrier leads to an increase of the protein concentration of the aqueous humor and thereby also of the aqueous flare density This density can be roughly estimated with the simple technique of focal illumination according to Gullstrand and described in ophthalmological textbooks (*Duke Elder* 1962) Determination of the course of variation of the aqueous flare density however requires an objective and sensitive method adapted for repeated measurements

In the present investigation a photoelectric device (*Dyster Aas & Krakau* 1963) was used to study the time course of the effect of endotoxin on the protein content of the aqueous humor to obtain a dose response relationship and to compare the flare provoking effect of endotoxins with the previously described effect of α melanocyte stimulating hormone and a minimal infrared trauma (*Dyster Aas & Krakau* 1965 *Dyster Aas* 1965)

Requests for reprints should be addressed to *H Fritz* Institute of Medical Microbiology Solvegatan 23 223 62 *Lund* Sweden

Acknowledgements *Mrs Monica Sthål* for skilful laboratory assistance The investigation was supported by grants from *Herman Järnhardts Stiftelse* and from *Kungl Fysiografiska Sallskapet i Lund*

MATERIAL AND METHODS

Rabbits pigmented and of either sex aged more than 6 months and weighing 2 5-4 0 kg (mean about 3 kg) were used The animals were fed on pellets and water

Endotoxins from *Salmonella abortus equi* (Difco 3172) and *Escherichia coli* (O 111 B4 Difco 3192) were commercial products prepared according to Westphal (*Westphal et al* 1952 *Westphal et al* 1958) Endotoxin from *Proteus mirabilis* was kindly supplied by Prof G Weibull University of Lund and prepared by him according to Westphal Endotoxin from *Salmonella enteritidis* (Ribi 6-195) was kindly supplied by Prof F Ribi Hamilton Montana USA and prepared according to *Ribi et al* (1961)

The endotoxins were dissolved in distilled water to a concentration of 50 µg per ml Tests for sterility yielded no growth Dilutions to 0 05-50 0 µg per ml pyrogen free physiological saline were prepared and used for injection One millilitre of the endotoxin solution was injected intravenously into an ear vein All together 144 injections were given to 91 rabbits i e 53 of the animals were used twice In these cases the period between the two injections was at least 3 weeks and in none of the animals was the endotoxin used the same on both occasions

The aqueous flare was measured with an electronic flash light lasting for a few milliseconds The flare density was quantitated photoelectrically as a peak value on an oscilloscope screen (*Dyster Aas & Krakau* 1963) With this method the normal relative protein concentration of the aqueous humor can be measured instantly without disturbing the blood aqueous humor barrier

Each value of the aqueous flare density used in the statistical analysis is the mean of 5 measurements on each eye Such a series of 10 measurements was made within 3 minutes and repeated at an interval of about half an hour until the maximum flare had been passed or if no increase occurred during 5-6 hours after the injection of endotoxin The aqueous flare value at a given time after the injection of endotoxin is the quotient of the actual flare value and the pre injection value determined in the same way The mean of this quotient for the two eyes (Q) is used in the statistical treatment The variation of the effect of the endotoxins with the dose used was assessed from the quotient in each experiment between the maximum flare value and the preinjection value (Q max)

The standard deviation of the aqueous flare recordings was less than 5 per cent A Q max value above 1 3 was regarded as a significant response and in the following referred to as a flare response

RESULTS

Maximum Aqueous Flare Response (Q max)

An intravenous injection of a sufficient amount of endotoxin was invariably followed by an increase of the aqueous flare A flare response was produced in 81 of the 144 experiments The relation between the dose size and the frequency of flare response is given in Fig 1 There was no significant difference in the frequency or the intensity of flare response between rabbits injected for the first and the second time respectively A dose of 0 05 or 0 1 µg used in 32 experiments produced no flare response A dose of 0 5 µg was the smallest that elicited a flare response in more than 50 per cent of the experiments It produced at least a tenfold increase of the aqueous flare density in 13 (39 per cent) of the 33 experiments with that dose In 17 experiments 5 0 µg was given and was followed by a flare response in all of them

The range of variation in Q max values obtained after the same dose of one endotoxin is given in Fig 2 For example injection of 0 5 µg

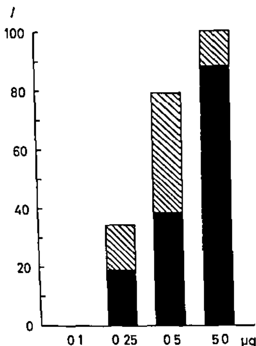


Fig 1

Percentage of experiments resulting in aqueous flare response to various doses of endotoxin not differentiated according to source The filled parts of the columns denote the frequency of at least a tenfold increase of the flare density 0 1 μg of endotoxin did not produce an aqueous flare response

of endotoxin from *Salmonella enteritidis* in 6 experiments produced no flare response in 2 but more than a tenfold increase of the flare density in 4 including 1 with a more than twentyfold increase Comparing the endotoxins by weight flare responses to doses of 0 25–1 0 μg were obtained with a significantly ($p < 0\ 01$) higher frequency with endotoxin from the Salmonella species than with the two other endotoxins This difference is even more pronounced when the tenfold or more increased flare densities are compared (Table I) Though the 2 Salmonella endotoxins were prepared by different methods they did not differ significantly from one another in the frequency with which they increased the density of the flare

0 25 μg of endotoxin from *Salmonella abortus equi* (48 experiments) produced a flare response in 6 of 12 rabbits and 0 5 μg in all the 9 rabbits injected (Fig 2a)

0 25 μg of endotoxin from *S enteritidis* (29 experiments) produced a pronounced aqueous flare response in 2 of 3 experiments and 1 0 μg in all 6 In 5 of these 6 the increase was at least tenfold (Fig 2b)

67 experiments were performed with endotoxin from *Proteus mirabilis or Escherichia coli* A dose of 0 25 μg produced a flare increase in

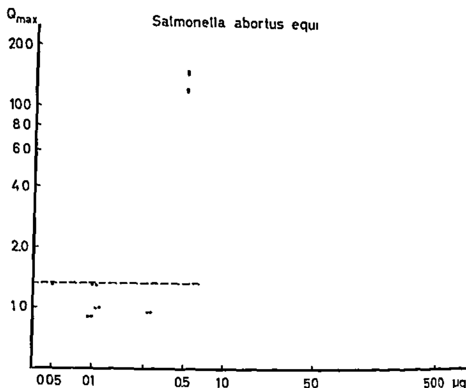


Fig 2a

The aqueous flare response (Q max) to various doses of endotoxin from *S abortus equi* (48 experiments) The dotted line (Q max 1 3) denotes the level above which the flare response is regarded as significant

only 1 of 11 experiments while injection of 0 5 or 1 0 μg in 36 experiments was followed by a flare response in 25 (69 per cent) including 9 where the increase was at least tenfold (Table 1 Fig 2 c d)

Course of the Aqueous Flare Response

As a rule the flare did not begin to increase until 1 to 1½ hours after the injection It reached its maximum about 5 hours after the injection The time course of the flare response to the median effective

TABLE 1

Frequency of Responses to Various Doses of Two Groups of Endotoxin

Dose µg	Frequency of flare responses		Frequency of at least tenfold increased flare density		Number of experiments
	Salmonella endotoxin	Escherichia or Proteus endotoxin	Salmonella endotoxin	Escherichia or Proteus endotoxin	
0 25	8/15 53 %	1/11 9 %	4/15 27 %	1/11 9 %	26
0 5	13/15 86 %	14/18 78 %	9/15 60 %	4/18 2? %	33
1 0	6/6 100 %	11/18 61 %	5/6 83 %	5/18 28 %	24

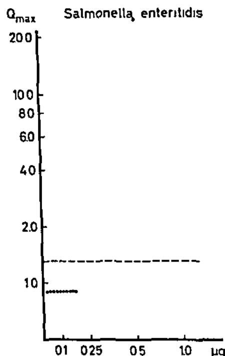


Fig 2b

he aqueous flare response (Q max) to various doses of endotoxin from *S enter* idis (29 experiments) *P mirabilis* (19 experiments) Fig 2c and *E coli* (48 ex eriments) Fig 2d, respectively The dotted line (Q max 1 3) denotes the level above which the flare response is regarded as significant

lose in the 144 experiments 0 5 µg is shown in Fig 3 Of the 33 ex periments performed with this dose there was a flare response in 27 (79 *per cent*) The mean Q values obtained in these 27 ex periments at different intervals after the injection show that a flare density of 7 to 8 times the normal was reached at a mean interval of about 4 hours and then remained high during the period of the examination Three rabbits given 0 5 µg of endotoxin from *S abortus equi* were ex amined also after 24 and 48 hours *the flare density* was *still 3 to 7* times the normal after 24 hours but after 48 hours the flare density dit not differ significantly from the normal 0 5 µg of endotoxin from *S enteritidis* produced higher mean Q values in responding animals than did 0 5 µg of the other endotoxins The difference was significant ($p < 0\ 01$) only on comparison with values obtained with the *E coli* endotoxin

DISCUSSION

A number of agents are known to be capable of producing an increased aqueous flare In the rabbit s eye a mild trauma such as infrared light

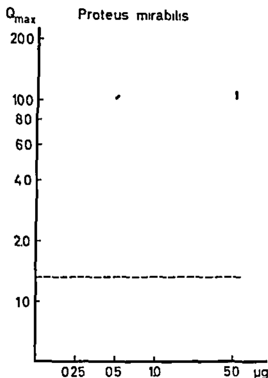

Q_max
Proteus mirabilis
200
100
80
60
40
2.0
10
0.25
0.5
1.0
5.0
µg

Fig 2c

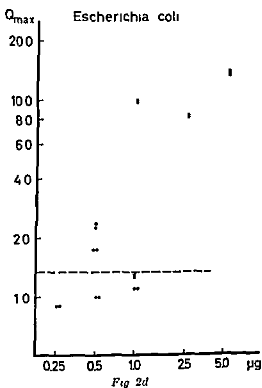

Q_max
Escherichia coli
200
100
80
60
40
20
10
0.25
0.5
1.0
2.5
5.0
µg

Fig 2d

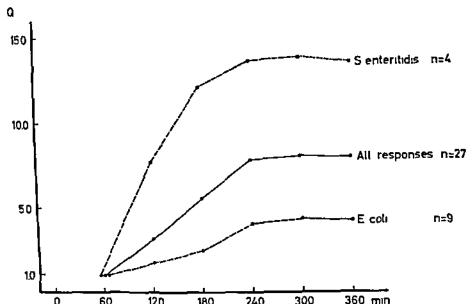
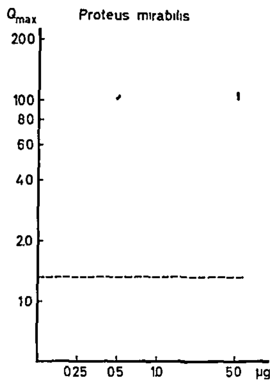


Fig 3

The mean course of the aqueous flare density in the 27 experiments with aqueous flare response to 0 5 μg of endotoxin The dotted lines denote the means of the 4 and 9 experiments respectively with response to 0 5 μg of endotoxin from *S enteritidis* or *E coli*

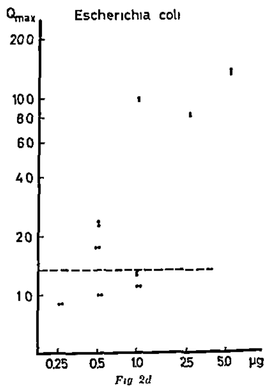
on the iris or puncture of the eye has a marked effect Corresponding ocular inflammatory effects have been observed after administration of various pyrogen protein solutions (*Seidy* 1930) after injection of killed streptococci (*Rosenow & Nickel* 1932) as well as after injection of coliform bacteria (*Barens & Nilson* 1941) Similarly α melanocyte stimulating hormone (MSH) may cause a considerable increase in the protein content of the aqueous humor owing to a histologically un specific disturbance in the blood aqueous humor barrier (*Dyster Aas Ehinger & Krakau* 1965)

Levene & Breinin (1959) usually employed a penlight in their examination for ocular inflammation following i v injection of endo toxin prepared by the Boivin method (*Boivin et al* 1935) Some of their rabbits were subsequently checked with a slit lamp for aqueous flare They reported such a flare in 95 per cent of the animals as part of an ocular inflammation described by *Ayo* (1942) and characterized by conjunctival and iris hyperaemia miosis photophobia lacrimation and increased coagulation of aqueous Levene & Breinin found an initial delay of 15 to 30 minutes before the development of the above signs of inflammation The intensity of the inflammation was highest between ½ to 5 hours Nothing was said about the smallest amount of endotoxin capable of producing the aqueous flare Their threshold



Qmax
Proteus mirabilis
200
100
80
60
40
20
10
025
05
10
50
μg

Fig 2c



Qmax
Escherichia coli
200
100
80
60
40
20
10
0.25
0.5
1.0
25
5.0
μg

Fig 2d

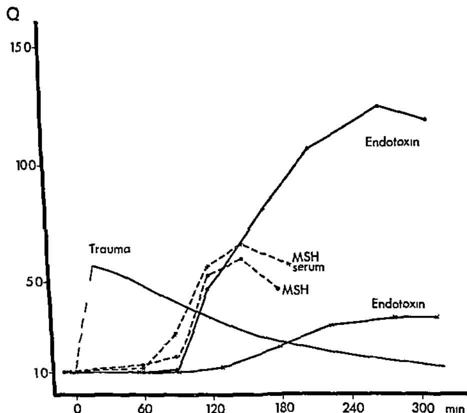


Fig 4

The course of the aqueous flare density following a minute trauma MSH or injection of serum from a rabbit treated with MSH compared with the flare response to endotoxin a selected experiment with a strong and a weak response respectively

other agents capable of producing an increased aqueous flare Such discrimination if possible may be useful in investigations on suspected endotoxin contamination of biologically active preparations Fig 4 compares the flare produced by endotoxin with the previously reported response to a minimal trauma (infrared light) and to intravenous injection of either MSH or serum from rabbits injected with MSH (*Dyster Aas & Krakau* 1968) In some respects the biological effects of MSH resemble those of the endotoxins MSH thus causes an increase of the body temperature and of the serum free fatty acids It is evident from Fig 4 that the aqueous flare response following infrared trauma or the injection of MSH can be separated from that to endotoxin The parallelism of the curves describing the effect of MSH and MSH activated serum on the aqueous flare support the theory that the response of the aqueous flare to MSH is secondary to some substance released in vivo Furthermore the endotoxins seems not to be involved in the effect of MSH or MSH activated serum

It might be mentioned that preliminary experiments with intravenous injection of up to 1 mg of endotoxin into rabbits have shown that serum from these endotoxin treated rabbits did not provoke a flare response when injected i v into other rabbits though the sera proved cytotoxic on monkey kidney cells (*Fritz & Nordenfelt* 1969)

The aqueous flare response to injections of endotoxin repeated within 24 and 48 hours respectively as well as the response in endotoxin tolerant rabbits will be the subject of a separate paper Preliminary experiments have shown that 0 1 μg of endotoxin an amount just smaller than half the single threshold dose is not sufficient to elicit an aqueous flare response even when injected twice 24 hours apart In the present study 53 rabbits were given a second injection of endotoxin more than 3 weeks after the first and of a preparation different in origin from that used before These rabbits on the second injection did not differ in response from fresh animals injected with the same endotoxin The specificity of the anti endotoxin antibodies hitherto recognized directed against the carbohydrate moiety of the endotoxin complex is dependent on the source of the endotoxin However in view of the fact that endotoxin in various stages of detoxification may persist for many weeks in the tissues it cannot be excluded that a greater number of experiments would have revealed some minor difference in the response between the above named two categories of animals

SUMMARY

A photoelectric device for studying the aqueous flare was used to quantitate and follow the course of the endotoxin effect on the protein content of the aqueous humor in the rabbit Intravenous injections of sufficient amounts of endotoxin from *S abortus equi S enteritidis, P mirabilis* and *E coli*, respectively were regularly followed by increased aqueous protein After a latency of 1–1½ hours the aqueous protein concentration increased and reached its maximum about 5 hours after the injection

The smallest amount of endotoxin producing an aqueous flare response was 0 25 μg i e about 0 1 μg per kg body weight In more than 50 per cent of the experiments a flare response was obtained after injection of 0 5 μg 5 μg was always followed by a marked response

Flare responses were obtained with a significantly higher frequency with endotoxin from the Salmonella species than with the two other endotoxins The method of preparation or origin of the endotoxin used did not influence the course of the flare density increase The course discriminated however the effect of endotoxin from that of a minute trauma injections of MSH or of MSH activated serum

REFERENCES

Ayo C A toxic ocular reaction I New property of Shwartzman toxins J Immunol 46 113–125 1943

Ayo C & Meyer K Protein content of the rabbits aqueous humor following in travenous injection of *E coli* toxin Proc Soc exp Biol & Med *51* 130–132 194?

Berens C & Nilsson E I Ocular conditions associated with coliform bacteria Clinical and experimental observations on coliform bacteria infections of the upper respiratory tract Arch Ophthal (Chicago) *26* 816–837 1941

Boivin A Mesrobeanu L Magheru G & Magheru A Recherches biologiques et chimiques sur l antigene somatique complet renferme dans quelques coli bacilles C R Soc Biol (Paris) *120* 1?76–1?79 1935

Burrows W Endotoxins Ann Rev Microbiol 5 181–196 1951

Duke Elder S System of ophthalmology vol VII Henry Kimpton London 1962 pp 246–260

Dyster Aas H K & Krakau C E T A photo electric instrument for measuring the aqueous flare in the intact eye Ophthalmologica (Basel) *146* 48–56 1963

Dyster Aas K Ehinger B & Krakau C E T Effect of the melanocyte stimulating hormone and of a minute trauma on the blood aqueous barrier in the rabbit eye. Acta Univ rsitatis Lundensis Sect II no ?0 Lund Sweden 1965 pp 1–7

Dyster Aas H K & Krakau C E T General effects of the melanocyte stimulating hormone in the rabbit Acta endocrinol (Kbh) 48 609–618 1965

Dyster Aas H K A comparison between the aqueous flare inducing effect of the α melanocyte stimulating hormone and a minimal trauma Acta Ophthal (Kbh) 43 30–46 1965

Dyster Aas H K & Krakau C E T Effects of the melanocyte stimulating hor mone and of an induced serum factor on eye permeability and serum level of free fatty acids in the rabbit Acta endocrinol (Kbh) *59* 487–496 1968

Frit H & Nordenfelt F Endotoxin induced cytotoxicity of rabbit serum Acta path microbiol scand 75 631–640 1969

Haskins W T Landy M Milner K C & Ribi F Biological properties of parent endotoxins and lipoid fractions with a kinetic study of acidhydrolyzed en dotoxin J exp Med *114* 665–684 1961

Levene R Z & Breinin G M Ocular effects of endotoxin A M A Arch Ophthal *61* 568–577 1959

Ribi E Haskins W T Landy M & Milner K C Preparation and host reactive properties of endotoxin with low content of nitrogen and lipid J exp Med *114* 647–6?3 1961

Ribi E Haskins W T Landy M & Milner K C Symposium on bacterial endo toxins I Relationship of chemical composition to biological activity Bac teriol Rev ?5 427–436 1961

Rosenow E C & Nickel A C Elective localization in determining the etiology of chronic u eitis Amer J Ophthal *15* 1–17 193?

Sr gpe al K F Retrobulbar blockade in endotoxin and horse serum induced uveal re p n e n rabb ts Graefes Arch Klin exp Ophthal *176* 263–276 1968

ton S il j A Exper mentelle Untersuchungen ub r die Wirkung der Reizkorper therapie auf Auge Klin Mbl Augenheilk 85 21–31 1930

Thomas L The role of epinephrine in the react ons produced by the endotoxins of gram negative bacteria I Hemorrhagic necrosis produced by epinephrine in the skin of endotoxin treated rabbit J exp Med *104* 865–880 1956

Watson D W & K n l B Immunologic l aspects of pyrogenic tolerance Landy and Br un (Ed) Bacterial endotoxins Institute of Microbiology Rutgers New Brun w k N J Quinn and B den Co Inc Rahway New Jersey USA 1964 pp 5??–53?

Westphal O Lüderit O & Biste F Uber die Extraktion von Bakterien mit Phe l Wasser *Z* Naturforsch *7b* 148–155 19 2

Westphal O Novotny A Luderit O Eich nberger E & Sch nholger G Die Bede tung der Lipoid komponente (lipoid A) fur die biologischen Wirkungen b kt r eller Fnd tox ne (Lipopolysaccaride) Pharm Acta Helv 33 401–411 1958

BRIEF REPORTS

IMMUNOSUPPRESSIVE ACTION OF L ASPARAGINASE STUDIED BY MEANS OF THE LOCALIZED HAEMOLYSIS IN GEL ASSAY (L H G A)

By *C V Muller Bérat*

This report deals with the action of a new type of anti leukemic compound an enzyme the L-Asparaginase (I) on the primary response to sheep erythrocytes in mice It is now demonstrated that cytostatics can exert their immuno depressive action through mitotic inhibition of the immuno competent cells though their point of impact on these cells is much less known It therefore seemed to us of interest to screen this compound not only on the haemolysins in the serum but also on a system where cellular proliferation is involved the L H G A thus emphasis was put on the time of administration of the drug in relation to the time of immunization A regimen of single high doses of L Asparaginase was used in the present experiment in order to avoid any interference with other phenomena like adaptation to the enzyme or formation of antibodies to the drug itself

Material and Methods

Female DBA/2 mice were immunized at day 0 with 10^8 sheep red cells I P Single doses of 8000 Units L Asparaginase/kg B W were injected Intra Peritoneally to 5 groups of 24 mice respectively at day —1 0 +1 +3 +7 in relation to the day 0 of Immunization Controls received sheep red cells only at day 0 Direct and developed plaques were determined by means of the L H G A as described by *Jerne et al* and modified by *Wortis & Dresser* (2 3) each spleen was assayed individually at day 4 for all groups and at day 5 and 7 for group 0 +1 +3 and Control care was specially taken to avoid clumps and to obtain a cell suspension fairly representative of the cellularity of the whole spleen to keep Ph buffer complement and type of sheep red cells identical throughout the experiments

Haemolytic titre in the serum was determined for all groups at day 3 4 5 7 after immunization Every indicated value is a mean of at least 5 mice

Results

A first screening at day +4 after immunization of the total number of PFC per spleen is shown in Fig 1

There is a significant depression in the number of direct and indirect plaques per spleen if L Asparaginase is given together with the antigen or shortly thereafter (24 hours) The effect is marginal on the 7 S plaques if the drug is given 24 hours before the antigen and it is not significant if given 3 days after the antigen It is also important to note that the plaques size in the group treated at day 0 was markedly smaller than that in control as known this reflects the quantity of antibody secreted at the cellular level The plaque assay was carried further on day 5 and 7 after immunization in order to see whether the depression induced in the group day 0 was long lasting and/or whether any enhancement could be observed (Table 1)

These results show that within this period of observation the peak of 19 S and 7 S in group 0 is postponed and remains depressed More experiments are needed

---

Received 19 xi 69 *from the Blood Bank Section of Leukemia and Transplantation Immunology Statens Seruminstitut 2300 Copenhagen S Denmark*

I wish to thank the *Bayer Pharmaceutical Company* for kindly providing the L Asparaginase mag scient *B Rubin* for providing the developing serum *I Bjorndal* *K Thomsen* and *P Nielsen* for skillful technical assistance Finally I thank dr *H Oettgen* for useful discussions

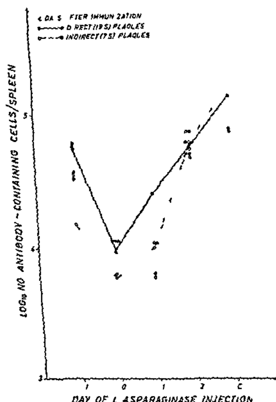


Fig 1

Effect of a single dose of 8000 u/kg B W of L Asparaginase on the number of antibody containing cells in the spleen 4 days after immunization Curve drawn through geometric means of the value in each group C mice given sheep red cells only

TABLE 1

Geometric Means of Total Direct 19 S and 7 S Indire t I F C per Spleen at Day 4 5 and 7 after Immuni ation

Group		0		+3		Control	
Type of P F C		19 S	7 S	19 S	7 S	19 S	7 S
Days after immunization	4	3 99	3 77	4 76	4 77	5 1?	5 19
	5	4 49	4 3?	4 89	5 88	4 17	4 96
	7	3 6	3 ?	3 85	5 12	3 7	4 5

t e nclude that there is an enhancement on the 7 S in group 3 At any rate there is no immuno depression in that group The serum haemolytic titres are shown in Table 2

The result of the haemolytic titre in the serum correlate with those observed at the cellular level Moreover it shows that at day 10 all groups are in this regard nearly similar to the controls

TABLE 2

Geometric Means of Values of Haemolytic Titre in all Groups at Day 3 4 5 6 7 and 10 after Immunization

	Groups	—1	0	+1	+3	+7	Control
	3	1	0 5	1	ND	ND	2
	4	3 3	1 5	1 7	5 8	ND	5 7
Days after	5	4 8	3 1	4 3	7	ND	6 7
immunization	6	6	4 3	5 3	7	ND	7
	7	7	5 3	4 9	7	6 9	7
	10	6 5	6 5	ND	6 4	6 4	6 3

Conclusion

From these data one can tentatively suggest that in this system L Asparaginase is effective in blocking the antibody synthesis at the level of the precursor cells at the time of the priming by the antigen and shortly thereafter (group 0 and group 1) This depression is compensated within 10 days after the immunization Both the 19 S and the 7 S plaques are affected within this period of observation Since no dissociation is observed on the level of these two classes of antibody which in the normal state are regulated by a reciprocal feed back mechanism it is *permitted to think that with this scheme of immunization and drug administration* L-Asparaginase acts on the two types of cell line or on the common cell giving rise to 19 S and 7 S There seems to be a wide range in the degree of protein synthesis inhibition induced by the enzyme on the cell on its way to DNA synthesis 1) total inhibition leading to an absence of replication 2) partial inhibition leading to a crippled progeny as expressed by the reduction in *plaque si e* observed in group 0 In contrast the absence of any significant depression obtained in group 3 i e when the drug was given in full exponential growth suggest that it does not act on the mitotic and probably not in our system on the DNA synthesis phase of the plaque forming cell We think that at dose level used in the present experiment L Asparaginase is not only active on leukemic lymphoblasts but also on a normal lymphoid population This is not surprising if one considers that metabolic defects specific of cancer cells are more quantitative than qualitative

At any rate we *believe that I Asparaginase deserves a clinical trial taking* advantage of its relative poor toxicity to the bone marrow A natural therapeutic choice will be auto immune diseases without liver involvement since it is on the liver that is expressed the toxicity of this drug In Transplantation Immunity we think that a special mention should be made in favour of the use of L Asparaginase in conditioning the host for a bone marrow transplantation by chemotherapy (as first used by *Santos & Owens*) and in the prevention of the graft versus host reaction

The drug can be given precisely at the time of antigen administration (the bone marrow inoculum) without being deleterious to the hemopoietic stem cell of the transplant However in all cases L Asparaginase appears more like a therapeutic complement to be used with other type of immuno suppressors in order to cover a broader spectrum of antimetabolic activity on the cell cycle of the target cells Work *is continued in this laboratory on heterochimeras and on an in vitro* assay using the immuno cyto adherence technique (4)

References 1 *Oettgen H P & Schulten H K* Klin Wschr 47 65–71 1969 — 2 *Wortis H H Taylor R B & Dresser D W* Immunology 11 603–616 1966 — 3 *Wortis H H Taylor R B & Dresser D W* Immunology 14 69–79 1968 — 4 *Bach J F Dardenne M & Fournier C* Nat 222 998 1969

Acta path microbiol. scand 77 753–765 1969

CELL MEDIATED AND HUMORAL IMMUNE REACTIONS AGAINST TUMOUR SPECIFIC AND VIRAL ANTIGENS IN RELATION TO ROUS SARCOMA VIRUS TUMORIGENESIS IN RATS

By *Nils Jonsson*

The presence of common tumour specific transplantation antigens (TSTA) in different tumours induced by the same virus is now a well established fact in a large number of viral tumour systems (for review see *Sjogren* (7)) Mammalian Rous tumours have a strong TSTA demonstrable by in vivo (4) or in vitro techniques (8) It seems of considerable interest to study immune reactions against such TSTAs in connection with the induction of primary tumours as such reactions would be expected to influence the development and growth of antigenic tumour cells The occurrence of such immune reactions in other experimental tumour systems has been reported by *Hellström et al* (1 2 3)

Material and Methods

Rats belonging to the inbred strain R/F were inoculated subcutaneously with 0 05–0 1 ml of a 1 5 suspension of SR RSV chicken sarcoma 3–6 days after birth About 65 per cent of the animals developed tumours at the site of inoculation after different intervals

Tumour bearing and negative animals were bled to death and lymph node cell (LNC) suspensions were prepared from the inguinal axillary cervical mesenteric and retroperitoneal lymph nodes by pressing the nodes through a stainless steel screen into Eagle s medium

Colony inhibition (CI) tests were performed with the sera (dilution 1 2) for detection of humoral immunity and on the LNC for detection of cell bound immunity against the Rous specific TSTA essentially according to the technique of *Sjögren & Hellström* (8) and *Hellstrom et al* (1 2 3) As target cells were used trypsinized allogeneic Rous rat tumour cells (RR 6690) since long established in vitro (2×10^3 cells per Petri dish) 12–24 hours later 2×10^6 LNC were added to the Petri dishes As has been shown by *Hellstrom et al* (3) allogeneic LNC donors can be used in CI tests to detect TSTA in different neoplasms if PHA is not added

Control sera derived from rats repeatedly pretreated 2–4 times with irradiated isografts from dimethylbenzantracene induced sarcomas and control LNC were taken from these rats or untreated rats No significant difference between these two control groups could be demonstrated SR RSV neutralization tests were performed according to *Rubin et al* (6)

Results

Tumour positive animals The results of the CI tests for cellular and humoral immunity in rats bearing primary tumours after different latency periods and after different tumour growth periods are collected in Table 1 LNC from 16 out of 20 rats significantly ($p < 0.01$) inhibited the colony formation by the target cells while 12 of the 20 sera had a significant effect No clear cut relation to the latency period or tumour growth time can be demonstrated Only one rat had developed demonstrable SR RSV neutralizing antibodies

Tumour negative animals The effect of LNC and sera from rats developing no

---

Received 20 xi 69 *from Institute of Pathology University of Lund Lund Sweden*

This work was supported by grants from the *Swedish Cancer Society* (Grant No 81 B69 02X) and *The Medical Faculty of the University of Lund* (Laenie Desbarres Lund)

Acta path microbiol scand 77 756–757 1969

TRANSMISSION OF *TOXOPLASMA GONDII*

Further Studies on the Morphology of the Cystic Form in Cat Faeces

By *J Chr Siim W M Hutchison* and *K Work*

In previous investigations on the transmission of *Toxoplasma gondii* in the faeces of experimentally infected cats it has been shown that

a) The floatings of faeces could transmit the infection to clean mice even in the absence of nematode eggs (6 1 3 4 9 8 2)
b) In these infective floatings oocysts were present in large numbers 1–[illegible] weeks after the cats had been fed with *Toxoplasma* tissue cysts (9 10)
c) There was a correlation between the number of the oocysts in serial dilutions of floatings and their infectivity for mice one single oocyst causing infection (9 10)
d) Microisolated single oocysts inoculated intraperitoneally into mice produced toxoplasma antibodies and tissue cysts in the brain (9 10)
e) When experiments were repeated with SPF cats free of protozoan *pathogens* identical oocysts were shed in the faeces The use of SPF cats excluded the possibility of natural coccidian infections (e g *Isospora felis*) being present in the cats prior to the feeding of chronically infected toxoplasmic tissues (5)
f) Finally after intraperitoneal inoculation of faecal floatings to mice trophozoites could be demonstrated in the exudate 11 days after inoculation free as well as intracellularly (7)

Morphology In unstained wet preparations the oocysts observed were ovoid measuring approximately $9 \times 14\ \mu$ having a cyst wall of uniform thickness After 3 weeks at room temperature two sporocysts had developed inside the oocyst measuring about $3 \times 7\ \mu$ Except for some granules no definite structures could be seen inside (9 10)

Further investigations on the morphology have provided additional information on the oocyst

Material and Methods

Floatings of faeces (no 5501) were obtained from cats infected with *Toxoplasma gondii* as previously described (9 10)

After centrifugation at 1500 r p m for 15 minutes the sediment was fixed in 10 per cent formalin and embedded in paraffin Sections $4–5\ \mu$ were stained with hematoxylin eosin

Results

In sections the oocysts are surrounded by an even structureless membrane which encloses two separate sporocysts Four sporozoites can be demonstrated in each of these sporocysts (Fig 1) These are elongate with one end pointed and the other *being more blunt In the pink cytoplasm* a dark blue nucleus is situated usually

Received 21 xi 69 from the Department of Toxoplasmosis and Viral Diseases State Serum Institute Copenhagen Denmark and the Department of Biology University of Strathclyde Glasgow Scotland

Aided in part by grants to Dr J Chr Siim from King Christian X Foundation US Public Health Service Research Grant Division (AI 01741) *Bethesda Md World Health Organisation Geneva* and *Cold Stores Foundation* and to *Strathclyde University by Medical Research Council Wellcome Foundation* and *World Health Organisation*



Fig 1

Section through an oocyst showing one of the two inner sporocysts Within this two of the four sporozoites can be seen distinctly Haematoxylin Eosin 2900 ×

nearer the blunt end The two single sporocysts are situated *in different planes* inside the oocyst It is therefore difficult to obtain sections showing both sporocysts and their content of four sporozoites

Naturally we are impressed with the similarities to the coccidian oocyst which this cyst exhibits It is remarkably similar to *Isospora bigemina*

Further cultural morphological cytochemical serological and immunological studies are required as well as investigations into the life cycle of the parasite, in order to finally establish its systematic classification and terminology

References 1 *Dubey J P* J Protozool *15* 773–775 1968 —2 *Frenkel J K Dubey J P & Miller N L* Science *164* 432–433 1969 —3 *Hutchison W M Dunachie J F & Work K* Acta path microbiol scand *74* 462 464 1968 —4 *Hutchison W M & Work K* Acta path microbiol scand 77 275–282 1969 —5 *Hutchison W M, Dunachie J F Work K & Sim J Chr* To be published —6 *Jacobs L* A[illegible] sitol 5 1–45 1967 —7 *Møller Ebba Schwartz* unpublished results 1969 —8 [illegible] *field H G & Melton M L* Science *164* 431–432 1969 —9 *Work K & Hutchison W M* Acta path micro biol scand 75 191–192 1969a —10 *Work K & Hutchison W M* Acta path microbiol scand 77 414–424 1969b

Addendum on Dec 8 1969 Since this paper was submitted *Hutchison* [illegible] *Sim & Work* Brit med J 4 806 1969 have observed profuse schizog[illegible] togony in the intestinal epithelium of the SPF cat which has been [illegible] the tissue cysts of *Toxoplasma gondii* The terminology used in [illegible] accordingly been changed to a coccidian basis

Acta path microbiol scand 77 758–760 1969

HETEROTRANSPLANTATION OF A HUMAN MALIGNANT TUMOUR TO NUDE MICE

By *Jørgen Rygaard* and *Carl O Povlsen*

Many attempts have been made to transplant human malignant tumours to various laboratory animals As a general rule the outcome of such attempts will be negative due to immune reactions

Changes in immune responsiveness may alter this result Thus neonatal thymectomy has been shown to increase survival of xenogeneic tumours (1) This report concerns serial transplantation of a human adenocarcinoma to the mouse mutant Nude suffering from recessive thymic aplasia (2 3)

Materials and Methods

Eight week old mice of the mutant Nude bred in Patologisk Anatomisk Institut Kommunehospitalet Copenhagen (3) were employed. Controls were phenotypically normal littermates presenting a normal fur These controls were inoculated to exclude a loss of antigenicity in the primary tumour Out of 4 malignant tumours tested (two anaplastic mammary carcinomas and two carcinomas of the colon) one has shown take

The tumour used was taken from a 74 year old woman (No 20 246/69 Surgical Department I Kommunehospitalet) The patient underwent abdominal surgery for a tumour of the sigmoid colon During the operation several big metastases of the liver were noticed The operative specimen was 16 cm in lenght and had a 3 cm broad circular tumour growing through all the layers of the sigmoid colon Microscopy (No 6792/69) showed a rather highly differentiated mucoid producing adenocarcinoma with total penetration of the wall 15 minutes after removal a cube of tissue measuring 5 × 5 × 5 mm was excised from the serosal side of the tumour under sterile precautions The cube was minced with a pair of scissors in 5 ml of Tissue Culture Medium 199 (Glaxo) Using a 12 G canula 0 5 ml of the suspension obtained was injected subcutaneously in the lateral abdominal wall in 3 Nudes and 3 controls

On the 21st day after inoculation parts of the two biggest tumours developed were removed for microscopical examination and serial transplantation A small block of this tumour tissue (2 × 2 × 2 mm) was implanted subcutaneously in the lateral abdominal wall in 6 Nudes and 1 control

After 14 days biopsies were taken from two of the Nudes of transfer No 2

Specimens for microscopical examination were fixed in formalin paraffin embedded and stained with hematoxylin eosin and van Gieson Hansen stain

Results

After 6 days a recognizable tumour appeared at the inoculation site in all the animals Whereas the tumours regressed slowly in the controls there was a rapid increase in the size of the tumours in the Nudes On the 40th day after inoculation tumour masses measured 10 × 20 × 25 mm in two of the mice and 5 × 5 × 12 mm in the third mouse of the first transfer In the controls no palpable tumour masses could be detected Microscopical examination showed no tumour growth

---

Received 23 xi 69 from Pathological Anatomical Institute Kommunehospitalet, Copenhagen and Autoimmune Laboratory Statens Seruminstitut Copenhagen Denmark

Requests for reprints should be addressed to *Dr J Rygaard* Autoimmune Laboratory Statens Seruminstitut Amager Boulevard 80 2300 Copenhagen S Denmark

We thank Mr *Oluf Rasmussen* who took care of the animals and Surgical Department I Kommunehospitalet for the tumour material



Fig 1

Top Section from operative specimen (KH 6792/69)
Bottom B opsy from transfer No 1 after 21 d ys (Hematoxylin eosin 140 ×)

The pattern of growth in transfer No 2 was the same as in the first inoculation Tumour growth thus appeared in all ino ulated Nudes In no case palpable meta tases were found and no ascites developed The animals have been kept alive for further investigations

The macroscopical appearance was that of a rather well circumscribed finely nodular solid tumour The cut surface as seen during biopsy was light grey and glary Histologically the tumour has remained constant during the transfers and has the characteristic of the primary tumour as to degree of differentiation con tents of connective tissue stroma and production of mucoid material

Discussion and Conclusions

Earlier attempts at heterotransplantation of tumours have shown that a successful outcome depends on transplantation to an immunologically unresponsive location in a normal animal *e g* the hamster cheek pouch or the anterior eye chamber in various species or transplantation to an immunologically unresponsive animal *e g* after exposure to X ray irradiation cortisone treatment induction of immunological tolerance or neonatal thymectomy The Nude represents an analogue to the latter condition only that thymic aplasia would exclude any prenatal effect of the thymus on the organism Nor is there any risk of neonatal thymectomy being subtotal

The demonstrated serial growth of a human malignant tumour in the mouse mutant Nude is interesting mainly for two reasons

a) It confirms the concept of the immunological deficiency of this mutant and

b) it may provide useful tools for the study of tumour immunity and for evaluation of therapeutic agents

Summary

Serial growth of a human malignant tumour in a mouse mutant (Nude) is reported for the first time

References 1 *Grogan J B* & *Hardy J D* J surg Res *8* 7–9 1968 —2 *Pantlouris E M* Nature (Lond) *217* 370–371 1968 —3 *Rygaard J* Acta path. microbiol scand 77 761–762 1969

Acta path microbiol scand 77 761-76? 1969

IMMUNOBIOLOGY OF THE MOUSE MUTANT NUDE

Preliminary Investigations

By *Jørgen Rygaard*

Congenital thymic aplasia in the hairless mouse mutant Nude (2) has been described by *Pantelouris* (4) It is an autosomal recessive The morphology of the lymphoid tissues in the Nude has been studied by *de Sousa et al* (7) These workers found the development of thymus dependent areas (5) to be even more defective than that following neonatal thymectomy

The immediate importance of this mutant as to the understanding of thymic function is evident The present communication will describe preliminary studies in the immunobiology of the Nude mutant

The Nude mutant has been bred in the Pathological Anatomical Institute Kommunehospitalet Copenhagen since December 1968 Two heterozygous pairs carrying genetic markers (1) *Rex* in male mice and *Trembler* in female mice were obtained from the Institute of Animal Genetics Edinburgh Scotland The heterozygotes have been paired *inter se* to maintain a breeding nucleus For large scale production of experimental animals male heterozygotes *Rex/nu* have been paired with specific pathogen free inbred Balb C female mice and females from a s p f closed but not inbred stock of the NMRI strain

The latter combinations produced ample offspring In the F_1 generation all mice with the Rex marker have been excluded and non marked animals have been paired at random within the offspring of each heterozygous male. This breeding scheme has produced homozygous *nu/nu* in the F_2 generation in about 80 per cent of the matings This proportion could be expected from the known recombination frequency of the Rex marker (?) During the first 10 months about 230 Nudes have been b ed All animals used for experimentation were out of the F_2 generation Littermates will be homozygous +/+ or heterozygous +/*nu* No attempt has been made so far to distinguish these phenotypically identical animals when selecting controls Such discrimination may however prove necessary

The low leucocyte counts found by *Pantelouris* (4) and the microscopical appearance of the lymphoid tissues reported by *de Sousa et al* (7) have been confirmed

Heterotransplantation of rat skin grafts was performed in six 8 week old Nudes Donors were Wistar rats from a closed but not inbred s p f stock The split skin graft technique of *Muller Berat et al* (3) was used It is noticeable that no oedema occurred as opposed to findings in normal mice following heterografting The colour of the grafts turned slightly hyperaemic in some areas cyanotic Complete healing appeared in 6 to 8 days when rejection would normally be recognizable

Biopsies were taken on days 2 4 and 6 The inflammatory reaction as seen by microscopical examination was very slight the infiltrate consisting of few granulocytes and large mononucleated cells On the 6th day there was a complete union of rat and mouse epidermal layers with a serous crust on the surface The underlying narrow dermal gap was filled with proliferating fibroblasts with very few inflammatory cells

One mouse died on day 10 with symptoms of wasting On the 12th day four of the Nudes had a secondary Wistar rat skin graft The secondary grafts were all accepted as readily as were the primary grafts

The hairs of the grafts were lost about 10 days after the operations but a normally looking fur developed during the following 15 days

---

Received 22 xi 69 *from Pathological Anatomical Institute Kommunehospitalet Copenhagen and Autoimmune Laboratory Statens Seruminstitut Copenhagen Denmark*

Request for reprints should be addressed to *J Rygaard* Autoimmune Laboratory Statens Seruminstitut Amager Boulevard 80 2300 Copenhagen S Denmark

The Nude with a single Wistar graft lived for 85 days after the operation and died from wasting with the graft intact

The double grafted Nudes died after 40-60 days also with signs of wasting There was a terminal loss of hairs in both primary and secondary grafts This may be due to a slow rejection

Results of heterografting of a human malignant tumour are reported elsewhere (6)

Preliminary immunoelectrophoretic studies have shown constantly minimal immunoglobulin values in homozygous *nu/nu* as compared to both heterozygous +/*nu* and normal Balb C mice 3-4 weeks after heterotransplantation of Wistar rat skin immunoglobulin values are normal or increased The possibility has to be considered that the proteins found may actually be rat immunoglobulins due to chimerism induced by the rat skin graft(s)

Some immune deficiencies that could be predicted from the morphological abnormalities of the mutant Nude have thus been demonstrated The condition described shows similarities to known human immune deficiencies but no total correspondence Yet for reasons stated this mouse mutant seems to offer facilities for experimental approaches to some basic problems in immunobiology Steps have been taken to establish the mutant in pathogen free and germfree conditions as a basis for further investigations

Acknowledgements

I thank *N Muller Berat Inger Bjorndal Oluf Rasmussen* and *Bendt Mansa* for advice and assistance and also Dr *D S Falconer* and Dr *P C Roberts* Edinburgh for the breeding nucleus

References 1 *Carter T C & Falconer D S* J Genet *50* 307-323 1951 —2 *Flanagan S P* Genet Res *8* 295-309 1966 —3 *Muller Berat N Sakai A & Moulias R* Scand Symposium on Immunology Stockholm Sweden March 1969 —4 *Pantelouris E M* Nature (Lond) *217* 370-371 1968 —5 *Parrot D M V de Sousa M A B & East J* J exp Med *123* 191 1966 —6 *Rygaard J & Povlsen C O* Acta path microbiol scand 77 758-760 1969 —7 *de Sousa M A B Parrot D M V & Pantelouris E M* Clin exp Immunol *4* 637-644 1969